Angiopoietins in renal replacement therapy

Westendorp, Welmoet Hillegonda

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Welmoet Hillegonda Westendorp

Welmoet H Westendorp
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Promotores
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Prof. dr. R.J. Ploeg

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Paranimfen
P.M. Snijder
A.S. Frenay

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CHAPTER 1

Introduction

RENAL REPLACEMENT THERAPY AND THE ANGIOPOIETIN/ TIE2-SYSTEM

The last few decades, the incidence and prevalence of end stage renal disease (ESRD) have been increasing worldwide[1]. Many of these patients ultimately require dialysis or a renal transplant. The latter is associated with better outcomes compared with maintenance dialysis therapy and therefore the preferred renal replacement treatment option. It results in superior quality of life and reduced mortality compared to dialysis therapy[2-4]. In renal transplantation, grafts are retrieved from living, deceased brain death (DBD) and deceased cardiac death (DCD) donors. Although kidneys obtained from living donors (LD) show better function and graft survival after transplantation then those obtained from deceased donors, their availability is limited[5-7]. Therefore, deceased donors are the main source for transplantation and most donor kidneys used are derived from DBD donors. Although not fully elucidated, not only post transplantation factors, but also donor condition is of major importance for long-term kidney graft survival[5,7,8]. In the DBD donor, a cascade of detrimental hemodynamic, inflammatory, hormonal, and immunologic events are induced that lead to endothelial activation thereby negatively affecting the function and outcome of transplanted kidneys[7,9-13]. Endothelial activation and disturbances in endothelial barrier function are considered to play an important role in the underlying brain death processes[14]. An important regulatory system in regulation of the endothelial barier is the constitutive Angiopoietin/Tie-signaling pathway, which is considered an important system in maintaining vascular quiescence[15].

The Angiopoietin/Tie-system

In 1996, long after the discovery of vascular endothelial growth factor (VEGF), a second specific vascular endothelial family of growth factors was identified, named the Angiopoietins[16-18]. The distribution of the Angiopoietin receptors, Tie1 and Tie2, is restricted to the vascular endothelium[19]. The endothelium presents a large surface area for exchange of materials between blood and tissues and is the first intima lining exposed to invading circulating pathogens. Endothelial disturbance is critically involved in many processes such as control of vascular tone, inflammatory responses, permeability and blood coagulation[20]. The luminal surface of the endothelial cells is lined with a glycocalyx layer and is considered as an intravascular compartment that protects the vessel wall against pathogenic processes[21]. Endothelial dysfunction or activation occurs in many diseases associated with an increased cardiovascular risk[22,23]. The direct contact between endothelium and plasma and cellular blood components rapidly increases expression of endothelial adhesion molecules, recruitment of leukocytes and vessel permeability upon pro-inflammatory activation[24]. The importance of the Tie1 and Tie2 vascular endothelial receptor tyrosine kinases for vascular formation have been revealed by genetic

gain- and loss-of-function experiments which clarified the functional consequences of the Ang/Tie2-system[18,25,26]. Tie2 is a 140 kD receptor and has been demonstrated to be essential for the development and of the vasculature[27]. All four known angiopoietins are secreted glycoproteins of approximately 70 kDa[28]. Of these, Ang1 and Ang2 are the best characterized and transgenic mice studies demonstrated that both are essential for correct vascular formation[25,29]. Ang1 and Ang2 regulate endothelial cell survival, angiogenesis and maturation via opposing functions via paracrine agonizing (Ang1) or autocrine antagonizing (Ang2) Tie2 phosphorylation, as Ang2 competes with Ang1 to bind to the Tie2 receptor[30-32]. Both bind to the Tie2 receptor with similar affinity[33]. To maintain the quiescent endothelium, low-level constitutive Tie2 activation is thought be required[19,34,35].

Studies on the roles of the Ang/Tie2-system report an important contribution in controlling these processes[32,36,37]. The proportion between Ang1 and Ang2 regulates endothelial barrier function, vascular leakage and inflammation that develop in response to pathogens and cytokines[36]. Binding of Ang1 to the Tie2 receptor induces Tie2 phosphorylation, providing an anti-inflammatory signal to the endothelium and thereby leading to vessel stabilization, a quiescent, anti-inflammatory endothelial status. In healthy adults, Ang1 is expressed at relatively constant rates by pericytes and vascular smooth muscle cells[19]. Ang1 maintains the Tie2 receptor in an activated state and protects endothelial cells from undergoing apoptosis via the PI3'-kinase/Akt signal transduction pathway (figure 1)[38,39]. Expression of Ang1 is not restricted to the vasculature, it is also stored in large amounts within platelets[40].

In contrast, a competition of Ang2 by preventing Ang1 from binding to Tie2 induces inhibition of Tie2 signal transduction and facilitates impaired endothelial function, increased inflammatory responsiveness and vascular leakage[15,18]. Ang2 was identified by sequence homology to Ang1[30] and is almost exclusively produced by endothelial cells[41,42]. Ang2 is only weakly expressed in endothelial cells under physiological conditions. Endothelial storage granules, Weibel Palade bodies (WPB), store Ang2 and quickly release it into the systemic circulation upon pro-inflammatory stimulation[43,44]. The functions of Ang2 appear to be more complex and it is suggested to act in a context-dependent manner as agonist and antagonist of Tie2 signaling[26,29,45-47]. High Ang2 concentrations can induce Tie2 phosphorylation and activate a similar signaling pathway as Ang1, exerting an antiapoptotic effect[48]. In contrast to the well-established antagonistic roles of Ang2, these agonistic functions are less well established. Excess WPB exocytosis as a consequence of decreased nitric oxide availability, like e.g. in chronic kidney disease (CKD), increases Ang2 levels[49].

Previous clinical and experimental data indicate a pivotal role of Ang2-driven endothelial activation in the pathogenesis of vascular inflammation, atherosclerosis and critical illness[18,50-53]. Binding of Ang2 to Tie2 antagonizes Tie2 signaling and primes the endothelium to respond to pro-inflammatory cytokines[54]. These

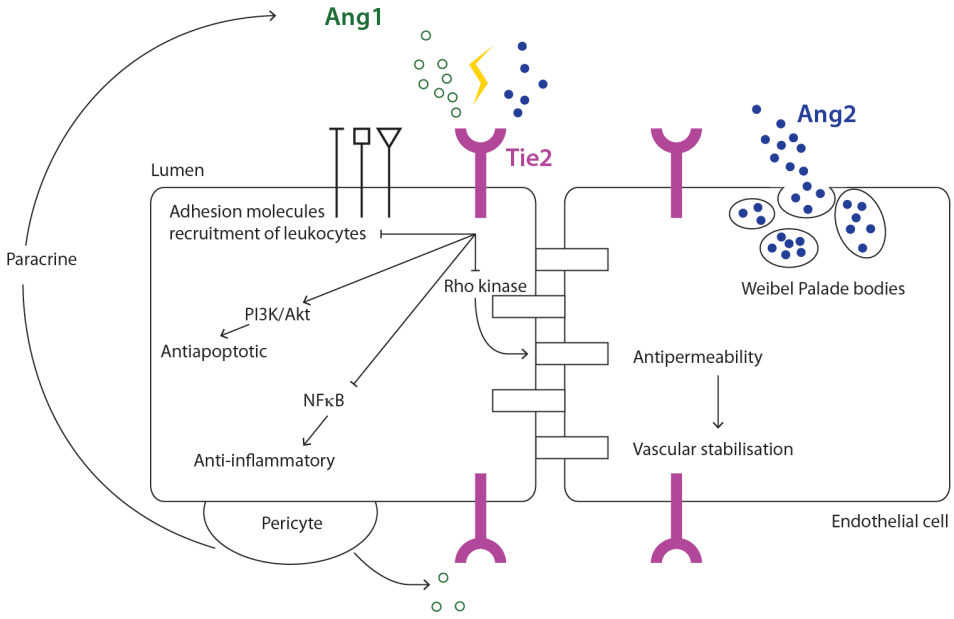

Figure 1. Overview of the Angiopoietin/Tie2-system at the endothelium. Adapted from van Meurs et al[52]. Pericytes attached to endothelial cells constitutively produce Ang1 in healthy individuals. Ang1 binds to the extracellular domain of the endothelial tyrosine kinase receptor Tie2 maintaining vascular quiescence. Binding to Tie2 activates the PI3K/Akt cell survival signaling pathway, thereby leading to vascular stabilization. Tie2 activation also inhibits the NF-κB-dependent expression of inflammatory genes, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin. In WPBs Ang2 is stored and rapidly released in an autocrine and paracrine fashion upon inflammatory stimulation. Ang2 acts as an antagonist of Ang1, stops Tie2 signaling and leads to vascular destabilization by sensitizing the endothelium to inflammatory mediators. Disruption of the protective Ang1/Tie2 signaling by Ang2 takes the cell-cell junctions via the Rho kinase pathway apart. This process causes capillary leakage and facilitates transmigration of leucocytes in inflammation. Abbreviations: Ang1: angiopoietin-1, Ang2: angiopoietin-2, Tie2: tyrosine kinase receptor Tie2, NFκB: nuclear factor kappa- light-chain-enhancer of activated B cells, PI3K: phosphoinositide-3 kinase, WPB: Weibel Palade body.

overactive responses are prevented by Ang1 mediated Tie2 phosphorylation, transducing anti-inflammatory and survival signals. After binding to Tie2, both Ang1 and An2 are released from the endothelium into the medium and are capable of binding to fresh cells, suggesting recycling of these ligands by endothelial cells[55].

Angiopoietins in renal transplantation

In both living and DBD donors, dynamic arteriovenous measurements over the reperfused kidney showed increased Ang2 release reflecting endothelial activation shortly after reperfusion while Ang1 was not released[56]. Angiopoietin levels and

function may reflect the immunogenic state of the donor organ and could be used as biomarker and intervention target to improve donor organ quality and outcome after transplantation. Prognostic significance of Ang2 has already been shown after trauma[57], sepsis[58] and acute pancreatitis[59]. In the general population and in clinical samples, elevated Ang2 levels predict cardiovascular events and mortality[60]. Moreover, circulating Ang2 is predictive of mortality in CKD patients[61] and upon renal transplantation (when measured after transplantation). A case-cohort study demonstrated that higher Ang2 levels are independently associated with increased all-cause mortality risk in renal transplant recipients (RTR)[62]. Even after successful renal transplantation, mortality rates are markedly higher compared to the general population[63-66] with cardiovascular disease (CVD) as the leading cause of death after renal transplantation underlining the critical role of the Ang/Tie2-system from donor to recipient[67,68].

In patients on dialysis, the prevalence of chronic inflammation, endothelial dysfunction, and accelerated atherosclerosis is high[69,70]. Elevated inflammatory factors are associated with an increased mortality risk[71]. The exact origin of chronic inflammation in dialysis patients remains unclear although the Ang/Tie2-system has been shown to play an important role in injury induced by CKD and dialysis. Previous studies have demonstrated an increase of circulating Ang2 with the progression of CKD which is predictive of mortality in these patients and correlates with severity of vascular disease in dialysis patients[50,61,72-74]. Because high Ang2 concentrations enhance endothelial responsiveness toward various cytokines and growth factors, Ang2 might act as an inflammatory sensitizer leading to vascular micro-inflammation in dialyis patients.

Modulating the Ang/Tie2-system

Investigating exogenous intervention in the Ang/Tie2-system might provide opportunities to maintain quiescent vascular endothelium, thereby preventing activation of further detrimental inflammatory effects. As competitive agonists and antagonists of Tie2, Ang1 and Ang2 represent the balance between resting and activated endothelium. The Ang/Tie2-system has already been studied as potential therapeutic target in various conditions in experimental models. Intravenous recombinant Ang1 administering alone was sufficient to significantly attenuate murine sepsis dysfunctions and survival time, most likely by preserving endothelial barrier function[75,76]. In mice, cartilage oligomeric matrix protein-angiopoietin-1 (COMP-Ang1), a variant of native Ang1, preserved renal tissue perfusion flow, microvascular permeability and decreased renal interstitial fibrosis after the ischemia-reperfusion injury[77]. COMP-Ang1 has also been reported to protect against endotoxemia-induced acute kidney injury (AKI) in mice[78]. The anti-inflammatory properties of Ang1 protected against the development of rat cardiac allograft arteriosclerosis[79].

Although therapy aimed at restoring Ang1 are promising in pre-clinical models, recent studies claim Ang2 to be the more dynamic player in the Ang/Tie2-system[53,60,80-86]. Attenuating pro-inflammatory Ang2 effects may therefore be another attractive target for therapeutic intervention in critical illness. Anti-Ang2 therapies have been studied in several preclinical models showing antiangiogenic effects in tumor-bearing rodents[84,87,88], less liver fibrosis in rats[89], preventing transplant ischemia-reperfusion injury and chronic rejection in rat cardiac allografts[85]. Phase III clinical trials using Ang2 inhibitors that have been performed to date provided promising results in malignancy[90-92].

The contribution of the Ang/Tie2-system in conditions wherein endothelial activation and dysfunction has a critical role, such as in renal replacement therapy, makes this system an interesting target to study. Appropriate treatment of patients on dialysis or the DBD donor by preventing endothelial destabilization by enhancing Ang1 mediated Tie2 phosphorylation or inhibiting Ang2 mediated signaling may be a tool to improve dialysis outcome, donor organ quality and subsequently transplant outcome.

AIM OF THE THESIS

The aim of this thesis is to elucidate on the alleged functional role of the Ang/Tie2-system before, during and after dialysis and renal transplantation.

Although the Ang/Tie2-system has been studied in CKD, studies investigating the role of angiopoietins in dialysis and renal transplantation have been limited. It came of interest to study in these conditions since endothelial dysfunction plays a considerable role. Therefore, we investigated whether angiopoietin levels in hemodialysis patients are associated with markers of inflammation and endothelial dysfunction, and if these levels associate with poor patient outcome in **Chapter 2**. In **Chapter 3**, we aimed to gain insight in the Ang2 levels in living donation and subsequently, how levels of the more dynamic Ang2 change from living donation through renal transplantation and reperfusion in the paired recipient. To further investigate the predictive capacities of Ang1 and Ang2 after renal transplantation, we prospectively studied the association of circulating Ang1 and Ang2 with the occurrence of graft failure and mortality in renal transplant recipients (RTR). We performed secondary analyses in recipients of a kidney derived from deceased donors. All these results are presented in **Chapter 4**. **Chapter 5** zooms in on the morphological and histopathological damage caused by brain death in renal transplant biopsies. We investigated the characteristics of pre-existent histopathological damage in both DBD and living donors. In **Chapter 6**, the endogenous role of Ang2 single nucleotide polymorphisms (SNPs) in the deceased donor and recipient on post-transplant outcome after renal transplantation was investigated. In **Chapter 7** an outline of the pathophysiology of brain death and

its detrimental effects on the potential donor kidney is presented. In the following chapter we focused on the contribution of the Ang/Tie2-system to the pathogenesis of brain death and the therapeutic potential of Ang1 and Ang2 as an endothelium-targeted agent in brain death donors. More specifically, in Chapter 8, we studied the effects of Ang1 and Vasculotide, an Ang1-derivate, in an experimental brain dead rat model to mimic the physiological setting of the brain death organ donor. The last few years, inhibition of the pro-inflammatory functions of Ang2 has gained considerable interest in both preclinical and clinical studies since Ang2 appears to be the more dynamic player in the Ang/Tie2-system. Therefore, we studied the protective potential of the Ang2 inhibitor AMG386 in our experimental brain dead rat model as well. All results are summarized and discussed followed by future implications in Chapter 9.

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CHAPTER 2

Angiopoietin-2 associates with markers of inflammation and cardiac damage and predicts cardiovascular events and mortality in dialysis patients

WH Westendorp
S Assa
H van Goor
R Westerhuis
SJL Bakker
HGD Leuvenink
CFM Franssen

Submitted

ABSTRACT

Background

Angiopoietin-1 (Ang1) and Angiopoietin-2 (Ang2) are involved in inflammation and vascular stabilization. Elevated Ang2 expression is caused by endothelial activation and may play a role in arteriosclerosis, cardiovascular disease (CVD) and mortality in chronic kidney disease (CKD). We studied the association of Ang1 and Ang2 with markers of inflammation, fluid overload, cardiac damage, clinical parameters and outcome in a prospective cohort of prevalent hemodialysis (HD) patients.

Study design and analytical approach

Plasma Ang1 and Ang2 were measured in 100 patients in a single HD session on four different time points: predialysis, at 60 and 180 min intradialysis and postdialysis (240 min). Cross-sectional analyses were performed through uni- and multivariable linear regression models and prospective analyses through uni- and multivariable Cox-regression models.

Candidate predictors

Ang1 and Ang2.

Outcomes

Cardiovascular events and all-cause mortality.

Results

Ang1 decreased from pre-HD to post-HD ($p=0.001$), whereas Ang2 levels rose markedly during the first hour of HD (from 2.6 (IQR 1.6-4.5) to 3.7 ng/ml (IQR 2.4-5.4), $p=0.005$) and subsequently decreased to values that were comparable with pre-HD values (2.5 ng/ml (IQR 1.9-3.6). For Ang1, no association with hsCRP, IL-6, TNFα, pentraxin-3, myeloperoxidase, pro-endothelin, ultrafiltration rate, Nt-pro-BNP and cardiac troponin T was observed. Similar analysis demonstrated associations with Ang2, independent of potential confounders (all $p<0.05$). At all time points, Ang2 levels associated with cardiovascular events (all <0.05). A greater intra-HD rise in Ang2 was associated with all-cause mortality (HR 1.2, 95%CI 1.2-1.2, $p<0.001$) independent of possible confounders. For Ang1, no such associations were found.

Conclusion

Ang2 is associated with inflammation and cardiac damage. Higher Ang2 levels are associated with a higher incidence of all-cause mortality and cardiovascular events in dialysis patients. HD treatment per se induces acute changes in angiopoietin levels suggestive of systemic endothelial activation. Greater intra-HD increases in Ang2 are associated with higher mortality. It remains to be studied whether intervention by Ang2 inhibitors attenuates the HD-induced angiopoietin-disequilibrium and improves outcome.

INTRODUCTION

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2), ligands of the Tie2 receptor and endothelial growth factors, are involved in stabilizing the vascular endothelium. Ang1 mediated Tie2 signaling promotes structural integrity of blood vessels and maintains quiescent endothelium[1,2]. Ang2 is classically considered as a Tie2 antagonist, counteracting the stabilizing effects of Ang1 in a dose-dependent manner and priming the endothelium to respond to exogenous pro-inflammatory stimuli[3,4]. An abnormal Ang1/Ang2 ratio, with downregulation of Ang1 and upregulation of Ang2 has been observed in CVD[5-7]. In clinical studies, elevated Ang2 levels were associated with peripheral artery disease[8] and heart failure[9,10]. In the general population, Ang2 levels predict cardiovascular morbidity[11]. Moreover, Ang2 expression is increased in plaque neovascularization, supporting the notion that endothelial activation is involved in the pathogenesis and progression of vascular inflammation and atherosclerosis[12,13].

Accelerated atherosclerosis in patients with CKD is still incompletely understood, but accumulating evidence suggests that Ang1 and Ang2 are important factors. Ang2 levels are elevated in patients with chronic kidney disease (CKD) and have a positive correlation with albuminuria and predict long-term mortality in this population[14-16]. In patients on hemodialysis (HD), Ang2 levels are markedly elevated and correlate with the severity of atherosclerotic lesions[12,15,17].

Ang1 is mainly synthesized in periendothelial cells, including vascular smooth muscle cells, pericytes and astrocytes. In adult vessels, Ang1 is constitutively expressed[18], while Ang2 expression is only observed at sites of active vascular remodeling and neoangiogenesis. Following endothelial activation, Ang2 is instantly released by exocytosis of endothelial Weibel Palade bodies (WPB)[19-21].

Although HD is life-saving in patients requiring renal replacement therapy, the HD procedure itself may contribute to the chronic inflammatory state of these patients. HD is capable of inducing various inflammatory pathways, mainly as a result of contact between the blood and the extra-corporal system, regardless of the dialyser type[22-24]. During HD, leukocyte activation is evidenced by increases in rapid-reacting cytokines like pentraxin-3[25], increased leukocyte transcript levels of several pro-inflammatory cytokines such as TNF-α and IL-8[26] and a rise in myeloperoxidase (MPO) levels during HD[27]. The HD-related systemic inflammation and oxidative stress may well contribute to the endothelial dysfunction of HD patients[28,29]. Ang2 might be a mediator and/or marker for inflammation, endothelial function, and accelerated atherosclerosis in dialysis patients. Furthermore, Ang2 could be a potential new therapeutic target to improve outcome by reducing the endothelial response to the chronic inflammatory state in CKD patients as well as the acute HD-induced inflammation. This is highly relevant since it is possible to influence Ang2 levels via Ang2 inhibitors[30,31].

In this study we tested the hypothesis that angiopoietin levels in dialysis patients are associated with higher levels of markers of inflammation, endothelial dysfunction, volume overload and cardiac damage. Additionally, we studied whether the HD procedure itself affects angiopoietin levels and if pre-, intra- and postdialysis Ang1 and Ang2 are associated with outcome.

METHODS

Patients and Study Design

For this study, we analyzed Ang1 and Ang2 levels in stored plasma samples of patients that participated in a prospective observational single-center cohort study[25]. Adult HD patients (aged ≥18 years) from the Dialysis Center Groningen and the University Medical Center Groningen were eligible if they had been treated with HD for more than 3 months and were on a thrice-weekly dialysis schedule. A total of 109 out of 235 in-center patients signed written informed consent. For the current post-hoc analysis, plasma Ang1 and Ang2 levels were measured in patients in which there were complete data and sufficient plasma sample volumes to measure Ang1 and Ang2 (n = 100, 91.7%). The study was performed according to the Declaration of Helsinki and the study protocol was approved by the Institutional Review Board of the University Medical Center Groningen (METc 2008.343). The study was performed between March 2009 and March 2010. Plasma Ang1 and Ang2 were measured in December 2013. Patients were studied at the dialysis session after the longest interdialytic interval (3 days).

HD session length was 4 hours. Patient characteristics were assessed at study entry from medical records. Diabetes was defined as fasting blood glucose level >6 mmol/L or use of antidiabetic drugs. Hypertension was defined as predialysis systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg or use of antihypertensive drugs. Cardiovascular history was defined as any history of ischemic heart disease, congestive heart failure, coronary artery bypass graft, percutaneous coronary intervention, stroke, or peripheral vascular disease. Blood pressure and heart rate were measured before, during and after HD. Ultrafiltration rate was expressed in ml/kg/h by dividing the ultrafiltration volume by dialysis session length and target weight[32]. Equilibrated Kt/V was calculated from pre- and postdialysis plasma urea concentration according to the second-generation logarithmic Daurgirdas equation[33].

Dialysis settings

All patients were on bicarbonate HD with a low-flux polysulfone hollow-fiber dialyzer (F8; Fresenius Medical Care). Blood flow and dialysate flow rates were 250-350 and 500 ml/min, respectively. Dialysate temperature was 36.0°C in all patients. Dialysate composition was as follows: sodium 139 mmol/L; calcium 1.5 mmol/L;

magnesium 0.5 mmol/L; chloride 108 mmol/L; bicarbonate 34 mmol/L; acetate 3.0 mmol/L; and glucose 1.0 g/L. Potassium concentration was 1.0 or 2.0 mmol/L, depending on prevailing plasma potassium concentrations. We used constant ultrafiltration rate and dialysate conductivity. The water for hemodialysis complied with the requirements of the European Pharmacopoeia (<100 colony-forming units/ml; <0.25 endotoxin units/ml). Patients received a light meal at 60 minutes intradialysis. Patients received dialysis in a supine position, which excluded the effect of posture changes on blood volume.

Laboratory procedures

Blood samples were collected from the arterial line of dialysis circuit at the start of HD, 60 and 180 minutes intra-HD, and at the end of dialysis. Hematocrit, leukocytes, neutrophils, albumin, calcium, and phosphate were determined immediately. For the determination of cytokines, blood was centrifuged within 30 min of collection at 3500 rpm for 15 min. Supernatants were stored at -80°C until measurement. Prior to assay, samples were thawed and recentrifuged. Samples were analyzed at a single time point to eliminate inter-assay variability.

Plasma parameters

High-sensitive CRP (hsCRP) was measured with N latex CRP monoassay (Siemens Diagnostic, Newark, DE, USA). Pentraxin-3 and interleukin-6 (IL-6) were measured by quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, USA). Pentraxin-3 was measured because it responds rapidly to inflammatory stimuli and is considered as an appropriate marker for investigating inflammatory reactions that may occur during single dialysis sessions[34]. Tumor necrosis factor α (TNFα) was measured by Quantikine HS Human Immunoassay (R&D Systems, Minneapolis, USA). Myeloperoxidase, which reflects activation of leukocytes, was measured by ELISA (Hytest, Turku, Finland). Pro-endothelin was measured by novel sandwich fluoroimmunoassay (BRAHMS, Hennigsdorf/Berlin, Germany) using the automated system B.R.A.H.M.S KRYPTOR. Measurement of endothelin was based on competition with surface-bound recombinant endothelin (RayBiotech, Norcross, GA, USA) for binding to a specific antibody (RayBiotech). The amount of captured antibody was measured by HRP-labeled secondary antibody and subsequent substrate conversion. Plasma Ang1 and Ang2 levels were measured via enzyme-linked immunosorbent assay (ELISA) Duosets (R&D Systems, Minneapolis, USA). Concentrations of all biomarkers measured during and after dialysis were corrected for the effect of hemoconcentration according to Schneditz et al[35].

Clinical endpoints

The primary endpoint of this study was all-cause mortality during a follow up period of three years. The secondary outcome was cardiovascular events defined as the

occurrence of ischemic heart disease, congestive heart failure, coronary artery bypass graft, percutaneous coronary intervention, stroke, or peripheral vascular disease. Patients were censored at the time of renal transplantation. Data endpoints regarding survival and cardiovascular events were obtained from hospital charts. None of the patients was lost to follow-up.

Statistical analysis

Data are reported as mean ± SD (standard deviation) for continuous variables with normal distributions, median [interquartile range] for skewed variables, and number (%) for categorical data. The Kruskal-Wallis test was used to assess whether angiopoietin levels were significantly different among the four time points. Subsequently, significant differences of Ang2 between two time points were tested using the Mann-Whitney test. Differences between patients with an increase versus a decrease in angiopoietins during HD were analyzed with Chi-square test in case of dichotomous variables and Mann-Whitney in case of continues variables. Skewed data were normalized for analyses by natural-logarithm transformation (LN) transformation. The associations of Ang1 and Ang2 levels with various clinical parameters were analyzed with crude linear regression analysis (model 1), with adjustment for age, sex, dialysis vintage (model 2), and with additional adjustment for diabetes, cardiovascular history and ultrafiltration volume (model 3). Regression coefficients are given as standardized betas. The same models were used to perform prospective Cox regression analyses for the association of angiopoietins with all-cause mortality and cardiovascular events. In the analyses of intradialytic Ang2 changes, additional adjustment was performed for predialysis Ang2 level (model 4). In the analyses of intradialytic Ang2 change, hazard ratios are given per standard deviation multiplied with 100. Two-sided P value <0.05 was considered significant. Statistical analyses were performed with SPSS version 20 (SPSS Inc. Chicago, USA).

RESULTS

Patient characteristics and angiopoietin concentrations

Baseline characteristics of the 100 patients eligible for angiopoietin analyses are shown in table 1. The median (IQR) age was 66 (50-75) years. Twenty-four patients had diabetes and 81 used antihypertensive medication. Twenty-three patients had a history of cardiovascular events. The course of Ang1 concentrations during HD is shown in figure 1a. Ang1 gradually decreased from 2.8 ng/ml (IQR 1.8-5.0) predialysis to 2.7 ng/ml (IQR 1.8-3.9) at 60 min intra-HD (p=0.36), 2.6 ng/ml (IQR 1.6-4.0) at 180 min intra-HD (p=0.47) and 2.2 ng/ml (IQR 1.5-2.9) post-HD. Post-HD Ang1 levels were significantly lower compared with predialysis levels (p=0.001). The course of Ang2 concentrations is shown in figure 1b. Ang2 medians differed significantly among the four time points (p=0.001). Ang2 increased significantly

Table 1. Baseline patient characteristics and predialysis biomarker concentrations

	Patients on HD n = 100
Age, years	66 [50-75]
Males, %	67
Dialysis vintage, years	1.7 [0.7-4.0]
Diabetes, %	24
Hypertension, %	81
Predialysis SBP, mmHg	141 ± 25
Predialysis DPB, mmHg	81 ± 17
Heart rate, BPM	75 ± 14
Body mass index, kg/m^2	25 [23-28]
Body surface area, m^2)	1.9 ± 0.2
Previous cardiovascular events, %	23
Ultrafiltration volume, ml	2578 ± 773
Ultrafiltration rate, ml/kg/h	8.6 ± 2.6
Hematocrit, %	35 ± 3.7
Albumin, g/L	39 [37-40]
Calcium, mmol/L	2.3 ± 0.2
Phosphate, mmol/L	1.6 [1.3-1.9]
Kt/V	4.2 [3.8-4.9]
Angiopoietin-1, ng/ml	2.8 [1.8-5.0]
Angiopoietin-2, ng/ml	2.6 [1.6-4.5]
hsCRP, mg/L	6.9 [2.9-13.8]
Nt-pro-BNP, ng/L	4 [1.7-8.5]
Cardiac Troponin T, ng/L	46.3 [27-81.1]
Cardiac Troponin I, ng/L	0.02 [0.01-0.03]
Pentraxin-3, ng/ml	2.6 [1.6-4.2]
Myeloperoxidase, ng/ml	84.7 [73.1-98.3]
IL-6, pg/ml	6.1 [4-8.6]
TNFα, pg/ml	3.4 [2.8-4.2]
Pro-endothelin, pmol/L	275 [224-330]
Endothelin, ng/ml	40 [22.5-63]
HbA1C, %	5.7 ± 1

Data are presented as mean ± SD in case of normal distribution and as median [interquartile range] in case of skewed distribution. IQR, interquartile range; n, number; HD, hemodialysis; SBP, systolic blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; hsCRP, high-sensitive CRP; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; HbA1C, glycosylated hemoglobin.

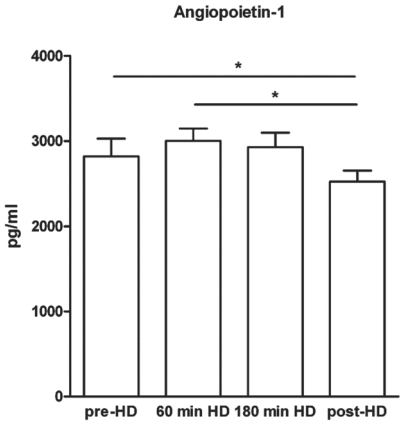

Figure 1a. Pre-, intra- and postdialysis Ang1 levels. Error bars indicate mean±SD of 100 patients. Postdialysis Ang1 levels were significantly lower compared with predialysis (p=0.001) and 60 min intradialysis (p=0.01).

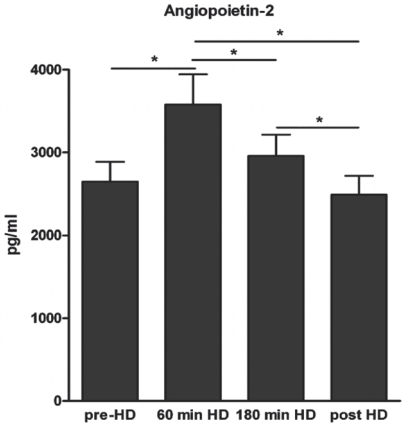

Figure 1b. Pre-, intra- and postdialysis Ang2 levels. Error bars indicate mean±SD of 100 patients. Ang2 levels increased significantly from predialysis to 60 min intradialysis (p=0.005). Pre- and post-HD Ang2 levels did not differ significantly (p=0.50).

from 2.6 ng/ml (IQR 1.6-4.5) predialysis to 3.7 ng/ml (IQR 2.4-5.4, p=0.005) at 60 min intra-HD. Subsequently, Ang2 decreased to 3.0 ng/ml (IQR 2.1-4.1) at 180 min intra-HD (p=0.02, compared to 60 min intra-HD) and 2.5 ng/ml (IQR 1.9-3.6) post-HD (p=0.04, compared to 180 min intra-HD). Pre- and post-HD Ang2 levels did not differ significantly (p=0.50).

Associations of predialysis Ang1 and Ang2 with clinical parameters and laboratory markers

Associations with predialysis Ang1 and Ang2 levels are shown in table 2. In the crude model, predialysis Ang1 levels were associated with higher systolic blood pressure (β 0.22 p=0.03), diastolic blood pressure (β 0.22 p=0.03) and cardiac troponin I (β 0.25 p=0.02). The associations with diastolic blood pressure and cardiac troponin I remained after further adjustment. Pre-HD Ang2 levels were associated with markers of inflammation, including hsCRP (β 0.21, p=0.04), IL-6 (β 0.40, p<0.001), TNFα (β 0.38, p<0.001), pentraxin-3 (β 0.33, p=0.001), myeloperoxidase (β 0.27, p=0.008), pro-endothelin (β 0.43, p<0.001), and cardiac damage (Nt-pro-BNP, β 0.52, p<0.001; cardiac troponin T, β 0.32, p=0.002) in the crude model. These associations remained materially unchanged after further adjustment.

Association between Ang1 and long-term outcome on dialysis

Median follow-up from baseline was 32.8 (IQR 15.9-36.5) months. During this follow-up, thirty-six (36%) patients died and thirty-five (35%) patients had at least one cardiovascular event. For Ang1, there was neither an association with all-cause mortality nor with cardiovascular events (supplementary table 1).

Association between Ang2 and long-term outcome on dialysis

The multivariate association of plasma Ang2 and outcome is shown in table 3. Predialysis Ang2 did not associate with mortality (HR 1.3, 95%CI 0.8-2.1, p=0.37, crude model). However, higher Ang2 levels were associated with higher mortality at 180 min intra HD (HR 4.5, 95%CI 1.9-10.5, p=0.001) and post-HD (HR 4.6, 95%CI 2.1-10.3, p<0.001), independent of age, sex, dialysis vintage, diabetes, cardiovascular history and ultrafiltration volume (model 3). Higher Ang2 levels were associated with a significantly higher incidence of cardiovascular events at all time points: pre-HD: HR 1.8, 95%CI 1.1-3.2, p=0.04, 60 min intra-HD: HR 1.9, 95% 1.1-3.4, p=0.03; 180 min intra-HD: HR 3.5, 95% 1.7-7.2, p<0.001 and post-HD: HR 3.3, 95%CI 1.7-6.4, p=0.001 in the fully adjusted model (model 4).

Association between the intradialytic change in Ang1 and Ang2 and long-term outcome on dialysis

As Ang1 levels decreased significantly from pre- to post-HD, we analyzed whether Ang1 change during the HD session was predictive of outcome. The change in Ang1 was not predictive of all-cause mortality or cardiovascular events (supplementary table 2). In patients with an Ang1 increase during HD (n=64), Ang1 rose from 1.9 ng/ml (IQR 1.4-2.5) pre-HD to 2.5 ng/ml (IQR 1.9-3.7) post-HD. In patients with a stable or decrease in Ang1 levels (n=36), Ang1 decreased from 3.6 ng/ml (IQR 2.3-5.4) pre-HD to 2.1 ng/ml (IQR 1.4-2.9) post-HD. All-cause mortality (Log-rank test p= 0.13) and cardiovascular events (Log-rank test p=0.29) did not differ between the 2 groups.

Table 2. Regression coefficients for the association of predialysis plasma Ang1 and Ang2 with various clinical and laboratory parameters in 100 hemodialysis patients

	Model 1		Model 2		Model 3	
Dependent variable	β	p	β	p	β	p
Angiopoietin-1*						
SBP (mmHg)	0.22	**0.03**	0.21	0.05	0.20	0.06
DBP (mmHg)	0.22	**0.03**	0.26	**0.02**	0.28	**0.02**
Heart rate (beats/min)	0.05	0.60	0.06	0.60	0.06	0.60
Ultrafiltration rate (ml/kg/h)	-0.05	0.62	-0.06	0.53	-0.06	0.55
Ultrafiltration volume (L)	-0.11	0.28	-0.09	0.30	-0.09	0.30
HbA1C (%)*	-0.008	0.94	-0.02	0.84	-0.02	0.88
hsCRP (mg/L)*	-0.03	0.78	-0.02	0.88	-0.03	0.78
IL-6 (pg/ml)*	-0.11	0.30	-0.11	0.29	-0.10	0.36
TNFα (pg/ml)*	0.01	0.95	-0.009	0.94	-0.02	0.88
Nt-pro-BNP (ng/ml)*	0.12	0.25	0.11	0.31	0.14	0.20
Cardiac troponin T (ng/L)*	0.12	0.23	0.17	0.15	0.24	0.05
Cardiac troponin I (ng/L)*	0.25	**0.02**	0.26	**0.03**	0.29	**0.02**
Pentraxin-3 (ng/ml)*	-0.01	0.91	-0.02	0.83	<0.001	0.99
Myeloperoxidase (ng/ml)*	0.19	0.07	0.19	0.07	0.19	0.08
Pro-endothelin (pmol/L)*	0.02	0.88	-0.001	0.99	0.03	0.81
Endothelin (ng/ml)	0.07	0.49	0.06	0.56	0.05	0.66
Angiopoietin-2*						
SBP (mmHg)	-0.03	0.74	-0.05	0.63	-0.06	0.60
DBP (mmHg)	-0.07	0.50	-0.05	0.67	-0.05	0.65
Heart rate (beats/min)	0.13	0.23	0.15	0.15	0.14	0.18
Ultrafiltration rate (ml/kg/h)	0.16	0.11	0.20	**0.03**	0.20	**0.04**
Ultrafiltration volume (L)	0.08	0.44	0.15	0.09	0.14	0.12
HbA1C (%)*	0.07	0.52	0.08	0.51	-0.03	0.84
hsCRP (mg/L)*	0.21	**0.04**	0.23	**0.04**	0.22	**0.04**
IL-6 (pg/ml)*	0.40	**<0.001**	0.39	**<0.001**	0.37	**0.001**
TNFα (pg/ml)*	0.38	**<0.001**	0.38	**0.001**	0.38	**0.001**
Nt-pro-BNP (ng/ml)*	0.52	**<0.001**	0.54	**<0.001**	0.54	**<0.001**
Cardiac troponin T (ng/L)*	0.32	**0.002**	0.39	**0.001**	0.38	**0.001**
Cardiac troponin I (ng/L)*	0.19	0.08	0.21	0.08	0.18	0.14
Pentraxin-3 (ng/ml)*	0.33	**0.001**	0.31	**0.003**	0.31	**0.003**
Myeloperoxidase (ng/ml)*	0.27	**0.008**	0.27	**0.01**	0.26	**0.01**
Pro-endothelin (pmol/L)*	0.43	**<0.001**	0.47	**<0.001**	0.45	**0.001**
Endothelin (ng/ml)	0.07	0.49	0.04	0.73	0.08	0.48

*Natural-logarithmic (LN) transformed for analyses. Coefficients are provided as standardized betas.
SBP, systolic blood pressure; DBP, diastolic blood pressure; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; hsCRP, high-sensitive CRP; HbA1C, glycosylated hemoglobin.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume (except in case of ultrafiltration volume and ultrafiltration rate as dependent variable)

Table 3. Cox regression analyses for prediction of outcome based on Ang2 levels

	All-cause mortality											
	Ang2 pre-HD*			Ang2 60 min intra-HD*			Ang2 180 min intra-HD*			Ang2 post-HD*		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Model 1	1.3	0.8-2.1	0.37	1.5	0.9-2.7	0.12	2.2	1.1-4.5	0.02	2.4	1.3-4.5	0.005
Model 2	1.4	0.8-2.4	0.22	1.5	0.9-2.5	0.16	2.6	1.3-5.3	0.008	3.0	1.5-5.7	0.001
Model 3	1.6	0.9-2.8	0.14	1.6	0.9-2.8	0.10	4.5	1.9-10.5	0.001	4.6	2.1-10.3	<0.001
	Cardiovascular events											
Model 1	1.9	1.2-3.3	0.01	2.3	1.3-4.2	0.004	3.4	1.8-6.3	<0.001	3.5	2.0-6.2	<0.001
Model 2	2.1	1.3-3.7	0.005	2.3	1.3-4.0	0.005	3.5	2.0-6.3	<0.001	3.7	2.1-6.3	<0.001
Model 3	1.8	1.1-3.2	0.04	1.9	1.1-3.4	0.03	3.5	1.7-7.2	<0.001	3.3	1.7-6.4	0.001

*Ang2 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. Ang2: angiopoietin-2, CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume

Table 4. Cox regression analyses for prediction of outcome based on the intra-HD change in Ang2 concentration

	Δ% Ang2 from pre-HD to post-HD*					
	All-cause mortality			Cardiovascular events		
	HR	95% CI	p	HR	95% CI	p
Model 1	1.2	1.2-1.2	0.008	1.2	1.2-1.2	0.95
Model 2	1.2	1.2-1.2	0.01	1.2	1.2-1.2	0.95
Model 3	1.2	1.2-1.2	0.006	1.2	1.2-1.2	0.96
Model 4	1.2	1.2-1.2	<0.001	1.2	1.2-1.2	0.49

*Concentrations were corrected for the effect of hemoconcentration. Hazard ratios per standard deviation multiplied with 100. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume
Model 4: as model 3 + adjusted for predialysis Ang2

Since the associations between higher Ang2 levels and higher all-cause mortality and cardiovascular events were stronger in the second half of the HD session, we also analyzed whether the change in Ang2 during HD was predictive of outcome. As shown in table 4, a greater intradialytic rise in Ang2 was associated with higher all-cause mortality in all models (HR 1.2, 95%CI 1.2-1.2, p<0.001). For cardiovascular events, no such association was found (table 4). In patients with an Ang2 increase (n=54), Ang2 rose from 1.9 ng/ml (IQR 1.2-3.2) pre-HD to 2.5 ng/ml (IQR 1.6-3.8) post-HD. In the remainder of patients, Ang2 levels were stable or decreased. In these patients, Ang2 levels decreased from 3.6 ng/ml (IQR 2.6-5.5) pre-HD to 2.5 ng/ml (IQR 2.0-3.5) post-HD. Patients whose Ang2 levels rose during HD had higher ultrafiltration volume (p=0.04) but lower predialysis Nt-pro-BNP levels (p=0.01) and lower predialysis Ang2 levels (p<0.001) compared with patients whose Ang2 levels decreased during HD (supplementary table 3).

DISCUSSION

The main findings of this study are that Ang2 levels are significantly associated with markers of inflammation, fluid overload and cardiac damage and that higher Ang2 levels are associated with a higher incidence of all-cause mortality and cardiovascular events. HD treatment induced acute changes in angiopoietin levels suggestive of endothelial activation and greater intra-HD increases in Ang2 were associated with higher mortality.

Only a few studies have investigated angiopoietins in HD patients[15,36]. We found slightly lower pre-HD Ang1 and Ang2 levels compared with another adult dialysis population, possibly as a result of the use of different assays[37]. Compared to serum

Ang1 and Ang2 in children on dialysis, we measured somewhat lower Ang1 and Ang2 plasma levels, perhaps caused by *ex vivo* activation of platelets in serum tubes and intra-essay differences between plasma and serum[38,39]. We did not include a control group with normal renal function but David et al previously found that patients on hemodialysis have lower Ang1 and higher Ang2 levels compared with healthy individuals[40]. In only 2 previous studies, Ang2 levels were measured before and after HD showing that predialysis and postdialysis Ang2 levels did not differ significantly[41,40]. Our study confirms the results of these studies but for the first time it shows that Ang2 levels peak after one hour of HD and subsequently gradually decrease to values that are comparable with predialysis levels.

Interestingly, Ang2 levels associated with various prognostically unfavorable factors like inflammation (hsCRP, pentraxin-3, IL-6, TNFα, myeloperoxidase, pro-endothelin), fluid overload (ultrafiltration rate, Nt-pro-BNP), and cardiac damage (cardiac troponin I). This may suggest that Ang2 has a central role in the pathogenesis of accelerated arteriosclerosis in HD patients. Non-hemorrhagic and hemorrhagic atherosclerotic plaques release Ang2[42]. The finding that Ang2 levels are elevated in patients with diabetes, CKD and atherosclerosis and correlate with vascular inflammation and disease progression supports the notion that elevated Ang2 levels reflect endothelial dysfunction[3,7,43]. The pro-inflammatory role of Ang2 in cardiovascular disease and atherosclerosis, has been demonstrated in previous studies[12,15,36,44]. The significant association between circulating Ang2 and hsCRP we demonstrated is consistent with previous reports[16,45]. Additionally, we found a significant association between Ang2 and the pro-inflammatory markers Il-6, TNF-alpha, myeloperoxidase, and pentraxin-3. Higher levels of these inflammatory markers are associated with a strongly elevated risk of cardiovascular events and mortality in patients on hemodialysis[46-48].

Since patients with CKD including those on maintenance HD are characterized by endothelial dysfunction[49,50] and since endothelial dysfunction is a known cause of Ang2 release[51,52], it is tempting to speculate that elevated predialysis Ang2 levels reflect endothelial dysfunction. This may also explain the strong association between Ang2 and pro-endothelin levels that we found. We observed a remarkable increase in Ang2 levels during the first hour of HD. The HD procedure may acutely worsen endothelial function as has been shown in children and adults[49,50]. Thus, the early rise of Ang2 during HD may reflect the deleterious effect of HD on the endothelium. HD acutely induces an inflammatory response as evidenced by significant intradialytic increases in rapidly reacting proinflammatory cytokines such as pentraxin-3[29,53]. The HD procedure is also associated with leukocyte activation and degranulation, which results in an acute intra-dialytic aggravation of oxidative stress[50]. Together, the inflammatory response and oxidative stress probably result in worsening of endothelial function during HD[50,54]. The early intradialytic rise in Ang2 levels may well reflect WPB exocytosis of Ang2 as a result of HD-induced inflammation and oxidative stress[21].

Patients with cardiovascular disease are more likely to have higher circulating Ang2 levels[6,9] compared with healthy controls. In the general population, elevated Ang2 levels are associated with an increased cardiovascular events and mortality[11]. Circulating Ang2 has also been shown to correlate with time on dialysis, systolic blood pressure and carotid artery intima media thickness in children with CKD on dialysis[36]. In line with these findings, we found an independent significant association between elevated Ang2 and cardiovascular events at all time points in patients on hemodialysis. In patients with CKD, Ang2 is an independent predictor of mortality[55]. In this study, only at 180 min intra-dialysis and post-dialysis an independent association of Ang2 with mortality was found. Presently, we do not know whether the absence of an association of predialysis and 60 min intra-dialysis Ang2 levels is real or is caused by a lack of power.

Our study has several limitations. First, since it is an observational study, conclusions on causality cannot be drawn. Second, our study population was relatively small and was predominantly Caucasian. This limits the generalizability of this study. Strong points are that this is the first study of angiopoietins in relation to markers of inflammation and endothelial function and outcome. These markers were not only measured before, but also during and at the end of HD and were corrected for hemoconcentration.

The high prevalence of chronic inflammation, atherosclerosis and increased mortality risk in dialysis patients renders the study of potential therapeutic targets highly relevant. Pharmacological Ang2 blockade that targets the angiopoietin/Tie2-system might potentially improve outcome in HD patients. Various studies have shown that it is possible to inhibit Ang2-induced Tie2 phosphorylation by antibodies in pre-clinical studies[30,31,56,57]. A peptibody, inhibiting the interaction between the Tie2 receptor and Ang1 and Ang2 was the first to enter a phase III clinical trial demonstrating promising results[58].

The present study shows that Ang2 levels increase significantly during the first hour of HD. The activation of the endothelial layer in hemodialysis is reflected by a disequilibrium in angiopoietins associating with inflammatory and cardiac damage markers. Plasma Ang2 is associated with all-cause mortality and cardiovascular events. The impact on patient outcome underlines the importance of understanding the responsible mechanisms concerning the Ang/Tie2-system. Clarifying this will possibly pave the way for therapeutic intervention studies in hemodialysis.

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Supplementary Table 1. Cox regression analyses for prediction of outcome based on Ang1 levels

	All-cause mortality											
	Ang1 pre-HD*			Ang1 60 min intra-HD*			Ang1 180 min intra-HD*			Ang1 post-HD*		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Model 1	1.02	0.56-1.85	0.96	0.75	0.36-1.57	0.44	0.82	0.45-1.50	0.53	0.87	0.40-1.90	0.73
Model 2	1.02	0.54-1.95	0.94	0.84	0.40-1.75	0.64	1.02	0.56-1.86	0.95	0.86	0.40-1.86	0.71
Model 3	1.03	0.54-1.98	0.93	0.82	0.38-1.77	0.62	1.13	0.59-2.13	0.72	0.86	0.40-1.85	0.71
	Cardiovascular events											
Model 1	0.99	0.54-1.85	0.99	1.01	0.49-2.06	0.99	1.33	0.71-2.49	0.37	0.87	0.42-1.83	0.72
Model 2	1.10	0.56-2.02	0.85	1.14	0.55-2.34	0.73	1.42	0.75-2.68	0.28	0.86	0.42-1.76	0.68
Model 3	0.95	0.50-1.78	0.86	0.91	0.43-1.93	0.81	0.99	0.47-2.07	0.97	0.89	0.42-1.87	0.75

*Ang1 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. Ang1: angiopoietin-1, CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume

Supplementary Table 2. Cox regression analyses on the prediction of outcome based on the intradialytic change in Ang1 concentrations

	Δ% Ang1 from pre-HD to post-HD*			Δ% Ang1 from pre-HD to post-HD*		
	All-cause mortality			Cardiovascular events		
	HR	95% CI	p	HR	95% CI	p
Model 1	1.59	0.78-3.25	0.20	1.50	0.73-3.09	0.27
Model 2	1.60	0.78-3.29	0.20	1.38	0.66-2.89	0.39
Model 3	1.56	0.75-3.24	0.23	1.48	0.67-3.26	1.48
Model 4	1.83	0.82-4.10	0.14	1.57	0.65-3.78	0.31

*Concentrations were corrected for the effect of hemoconcentration. Hazard ratios are associated with a 1-unit increase in each covariate. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume
Model 4: as model 3 + adjusted for predialysis Ang1

Supplementary Table 3. Baseline demographics and characteristics in patients with an intradialytic decrease versus those with an intradialytic increase in Ang2 concentration

	Change from pre- to post-HD		
	Ang2 decrease n = 46	Ang2 increase n = 54	p
Age, years	65 [53-75]	66 [49-75]	0.56
Males, %	67	69	0.90
Dialysis vintage, years	2.2 [0.7-4.0]	1.5 [0.6-3.9]	0.79
Diabetes, %	21	28	0.46
Hypertension, %	79	82	0.69
Predialysis SBP, mmHg	145 ± 27	138 ± 24	0.59
Predialysis DPB, mmHg	82 ± 19	80 ± 15	0.83
Heart rate, BPM	74 ± 14	76 ± 14	0.46
Body mass index, kg/m^2	25 [23-28]	25 [22-28]	0.86
Body surface area, m^2	1.9 ± 0.2	1.9 ± 0.2	0.64
Previous cardiovascular events, %	21	22	0.70
Ultrafiltration rate, ml/kg/h	8.1 ± 2.7	9.0 ± 2.5	0.09
Ultrafiltration volume, ml	2395 ± 745	2729 ± 776	0.04
Hematocrit, %	34 ± 3.3	35 ± 3.7	0.39
Albumin, g/L	40 [38-42]	39 [37-41]	0.65
Calcium, mmol/L	2.3 ± 0.1	2.3 ± 0.2	0.24
Phosphate, mmol/L	1.7 [1.4-2.1]	1.7 [1.3-1.9]	0.40
Kt/V	4.4 [3.9-4.7]	4.1[3.8-4.5]	0.18
Biomarkers			
Angiopoietin-1, ng/ml	3.9 [2-4.5]	2.3 [1.8-4.1]	0.05
Angiopoietin-2, ng/ml	3.6 [2.6-5.5]	1.9 [1.2-3.2]	<0.001
hsCRP, mg/L	6.9 [4-12.8]	6.9 [2.1-14.1]	0.82
Nt-pro-BNP, ng/L	6.3 [2.1-13.3]	3.3 [1.5-5.9]	0.01
Cardiac Troponin T, ng/L	53 [24.5-81.1]	44.7 [28.4-83.3]	0.90
Cardiac Troponin I, ng/L	0.02 [0.01-0.04]	0.02 [0.01-0.03]	0.71
Pentraxin-3, ng/ml	2.6 [1.6-4.2]	2.7 [1.8-4.6]	0.56
Myeloperoxidase, ng/ml	86 [74-101]	81.4 [71.2-97.4]	0.32
IL-6, pg/ml	7.2 [4-9.6]	5.9 [3.5-7.8]	0.34
TNFα, pg/ml	3.6 [2.8-4.6]	3.4 [2.8-4]	0.39
Pro-endothelin, pmol/L	273 [232-331]	280 [220-334]	0.86
Endothelin, ng/ml	34 [18-53]	42 [25-66]	0.09
HbA1C, %	5.5 ± 0.9	5.8 ± 1.1	0.17

Data are presented as mean ± SD in case of normal distribution and as median [interquartile range] in case of skewed distribution. IQR, interquartile range; n, number; HD, hemodialysis; SBP, systolic blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; hsCRP, high-sensitive CRP; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; HbA1C, glycosylated hemoglobin. P for difference was tested by the Mann-Whitney U test for continuous variables or Chi-square test for binary variables.

CHAPTER 3

Circulatory and renal angiopoietin-2 release in living donor renal transplantation

WH Westendorp
GJ Nieuwenhuijs-Moeke
NJ de Vries
PJ Ottens
H van Goor
RJ Ploeg
MMRF Struys
HGD Leuvenink

ABSTRACT

Background

The Angiopoietin/Tie2-system has been suggested to play an important role in the endothelial activation in renal transplantation. Little is known about baseline angiopoietin-2 (Ang2) levels throughout renal transplantation. Therefore, we aimed to define baseline Ang2 changes in living donor renal transplantation from donation to reperfusion in the recipient.

Methods

In a single center transplantation population, circulatory and arteriovenous Ang2 was measured in 53 matched living kidney donors and recipients.

Results

In the donor, plasma Ang2 increased significantly between preoperative levels and kidney retrieval (p=0.01). During reperfusion in the recipient, Ang2 levels were increased compared to Ang2 levels at time of donation but no arteriovenous differences were found. Plasma Ang2 decreased significantly at 2h postoperative compared to preoperative Ang2 in the recipient (p=0.003).

Conclusion

Circulating Ang2 is affected by renal transplantation, although Ang2 release from the kidney itself is not affected by reperfusion in this single center living kidney transplant population.

INTRODUCTION

The role of the endothelium in renal transplantation is considered increasingly important[1]. The key to long term allograft survival may lie in maintaining the endothelial cells, the inner lining of all blood vessels, in a quiescent state. Various effects during renal transplantation like endotoxemia and inflammation in the deceased brain dead (DBD) donor as well as ischemia/reperfusion injury (IRI) in the living and deceased donor, will activate the endothelium[2-5]. In both donor types, the activated endothelium forms an important mediator in the pathological processes negatively affecting patient and graft survival[6-8].

Endothelial homeostasis is regulated by the Ang/Tie2-system[9]. The endothelial cells are supported by pericytes which produce Angiopoietin-1 (Ang1)[10,11]. Ang1 and Angiopoietin-2 (Ang2) are the most important ligands of the tyroskine kinase receptor Tie2, which is mostly expressed by endothelial cells[12]. It is suggested Ang1 and Ang2 have opposite effects, with Ang1 inducing endothelial survival signals, inhibiting apoptosis and vascular inflammation and suppressing vascular/endothelial leakage[13,14]. By contrast, Ang2 acts as an Ang1 antagonist destabilizing the endothelium[15-17]. After endothelial activation by increased cytokine release, endothelial storage granules, Weibel Palade bodies (WPB), quickly release Ang2[18,19].

Studies on the role of angiopoietins in renal transplantation are limited. A study in a limited number of patients showed an increased Ang2 release after reperfusion in kidneys derived from living as well as deceased donors[20,21]. In rats an increase in Ang2 protein expression in the transplanted kidney after reperfusion in rats was shown[20,21]. Experimental studies on renal endothelial damage report that Ang1 overexpression improved renal function and blood flow after renal IRI in mice, as well as decreased influx of inflammatory cells and renal interstitial fibrosis[22]. In a mouse model of antiglomerular basement membrane glomerulonephritis, glomerular capillary loss was associated with reduced Ang1 and increased Ang2 expression, suggesting a relation between angiopoietin disbalance and endothelial cell loss[23]. In humans, besides the scope of renal transplantation, endothelial activation and dysfunction is a known cause of Ang2 release in chronic kidney disease and hemodialysis[24,25].

Although circulating angiopoietin levels have been studied in renal transplant recipients and small numbers of living and deceased kidney donors[21,26-28], little is known of the Ang2 response during a renal transplantation procedure. Studying plasma Ang2 in living donor kidney transplantation gives the opportunity to observe Ang2 levels in donors and recipients in the absence of profound systemic changes as demonstrated in deceased donors.

The aim of the present study was to define baseline Ang2 changes in living donor renal transplantation from donation to reperfusion in the recipient in a single center transplantation population. Results from the current study will provide crucial knowledge on physiological changes in Ang2 levels helping to interpret changes

found in deceased donation and transplantation. These baseline levels can be used as a best standard in human renal transplantation since living kidney donors are healthy individuals selected on the basis of absence of any disease. Furthermore, these baseline levels will provide a starting point for designing further intervention studies, modulating the Ang/Tie2-axis, as a potential strategy to improve donor organ quality and subsequently, transplantation outcome.

METHODS

Study population

For this study, we analyzed Ang2 levels in stored plasma samples of patients that participated the Volatile Anesthetic Protection of Renal transplants (VAPOR)-1 trial, a prospective randomized control trial on the effects of two different anesthetic regimens on renal outcome in living donor kidney transplantation (LDKT). Inclusion criteria were written informed consent, ≥18 years, and donation of the left kidney. Exclusion criteria were: right kidney donation, generalized central neurological disorder, donor-recipient couples from the ABO-incompatible program and altruistic donors. The Institutional Review Board approved the study protocol (METc 2009/334), which was in adherence to the Declaration of Helsinki. Between September 2010 and September 2012 125 LDKT were performed of which 88 involved the left kidney. Of that 88 couples 60 couples (68.2%) met inclusion criteria and gave written informed consent. Patients were randomly assigned to three groups according to the anesthetic regiment they received during the procedure. PROP if the donor and recipient received propofol based anesthesia, SEVO, if they received a sevoflurane based anesthesia and SERE if the donor received propofol and the recipient a sevoflurane based anesthesia. In the PROP group three patients were lost to follow up, two by violation of the surgical protocol and one patient died nine days post transplantation due to bleeding complications. In the SEVO group one patient was lost to follow up by violation of the immunosuppressive protocol. For this study we focused on Ang2 levels without division in the three groups. Therefore plasma of 53 donor-recipient couples was available for Ang2 and IL-6 measurement.

Operation and sample withdrawal

Kidney donation procedure was performed via the hand assisted laparoscopic (HAL) technique. Left gonadal vein which ends in the left renal vein was also dissected and retrieved. After explantation the kidney was immediately flushed and perfused with cold University of Wisconsin solution (Costorsol, Bridge to Life, USA) and stored on ice. In the recipient kidney transplantation was performed according to local standardized protocol. Prior to implantation a small sampling catheter was inserted in the gonadal vein as described by de Vries et al[29]. Immune

suppressive protocol was according to standard institutional guidelines. Patients received myophenolate mofetil (MMF) and cyclosporine/tacrolimus preoperatively at the ward and basiliximab and (methyl)prednisolon after induction of anesthesia. Post operatively they received an additional dose of basiliximab on day four and a protocol of MMF, methylprednisolon and ciclosporin/tacrolimus.
In donor and recipient pre-, per- and postoperative EDTA and citrate blood samples were withdrawn at standardized time points from an arterial line in the radial artery of the non-dominant/non-shunt arm. In the recipients additional samples were taken after reperfusion of the kidney via the catheter in the gonadal vein. These samples were taken simultaneously with systemic arterial samples at 30 sec, 5, 10 and 30 min after reperfusion. Two open kidney biopsies were performed, a cold biopsy when the kidney prior to implantation and a reperfusion biopsy approximately 45 minutes after reperfusion. Biopsies were performed using a Pro-Mag 2.2 Biopsy Gun with a 16-gauge needle (Manan Medical Products, USA) and subsequently stored in formalin and paraffin fixed until analysis. All samples were immediately placed on ice. Blood samples were centrifuged (1500g, 20 min, 4°C) and stored at -80°C until measurement. Prior to assays, samples were thawed and recentrifuged. Samples were analyzed in one batch to eliminate inter-assay variability.

Plasma measurements

Plasma levels of Ang2 and IL-6 were determined by enzyme linked immonusorbent assays (ELISA) according to manufacturers' instructions (R&D Systems, Minneapolis, USA). All samples were analyzed in duplicate and read at 450 nm using a microplate spectrophotometer (Victor3, 1420 multi-label counter, Perkin Elmer, USA). Serum creatinine was determined using an enzymatic essay on a Roche Modular chemistry analyzer (Roche Diagnostics, USA).

Clinical parameters

Data on donor and recipients' health status, medical history, renal function and medication were noted in a case record file as well as data about the procedure and postoperative period. Body weight and height were measured with participants wearing indoor clothing without shoes. BMI was calculated as weight divided by height squared (kg/m^2). Information on graft function (delayed graft function (DGF) and rejection) was retrieved from local digital patient records. DGF was defined as need for dialysis in the first week after transplantation other than immediately postoperative. Rejection was biopsy proven with decline in kidney function and the need of treatment.

Statistical analyses

Differences between arterial and venous samples were tested using the paired, nonparametric Wilcoxon test. Unpaired differences of Ang2 between two time

points were tested using the Mann-Whitney test. Graph error bars represent the SEM, unless otherwise stated. Normal distribution was tested using normal probability plots. Correlations were tested using Spearman's rho or lineair regression. Associations with graft function were assessed using Cox regression analysis. P<0.05 was considered significant. Statistical analyses were performed with SPSS version 20 (SPSS Inc. Chicago, USA).

RESULTS

Demographics of the adult patients undergoing donor nephrectomy and their matched recipients for living donor kidney transplantation are shown in table 1. Donor mean age was 53 ± 11 years and recipient mean age was 50 ± 13 years. As anticipated, the rate of DGF and rejection was low in this study population (<2%). Three patients developed DGF, nine patients showed an acute rejection episode during the two year follow up. As shown in table 2, no correlations between preoperative donor plasma Ang2 and clinical parameters were found.

Table 1. Demographics of 53 living kidney donor-recipient couples

Donor	
Living unrelated donor, n (%)	26 (49.1)
Age (years)	53 ± 11
Male sex, n (%)	24 (45.3)
BMI (kg/m^2)	26.9 ± 3.2
Duration of hospital stay (days)	6 [5-7]
IL-6 (pg/ml) after start anesthesia	2.3 [1.7-4]
Transplant demographics	
HLA mismatches (% of 0 mismatches)	7 (13.2)
Cold ischemia time (min)	177.4 ± 29.7
Recipient	
Age (years)	50 ± 13
Male sex, n (%)	24 (45.3)
BMI (kg/m^2)	25.1 ± 3.5
Duration of hospital stay (days)	17 [17-18]
Post-transplant parameters	
Serum creatinine 3 months after Tx (µmol/L)	131.1 ± 39.3
Delayed graft function (%)	3 (5.7)
Biopsy proven rejection in first 2 years after Tx (%)	9 (17)

Data are presented as mean ± SD in case of normal distribution and as median [interquartile range] in case of skewed distribution. BMI: body mass index, Tx: transplantation. Delayed graft function: the need for dialysis within <1 week after transplantation.

Table 2. Univariate analysis between systemic preoperative donor Ang2 and clinical parameters

Clinical parameter	ρ	P
Age (years)	0.07	0.60
BMI (kg/m^2)	0.23	0.10
Gender	-0.04	0.78
Cold ischemia time (min)	0.22	0.11
Duration of hospital stay (days)	0.02	0.90

ρ: Spearman's correlation coefficient, BMI: body mass index.

Table 3. Univariate analysis between systemic plasma Ang2 one day after transplantation and clinical parameters in the recipient

Clinical parameter	ρ	P
Age (years)	0.28	0.04
BMI (kg/m^2)	0.06	0.67
Gender	-0.05	0.72
Duration of hospital stay (days)	0.36	0.01
IL-6 1st day after Tx (pg/ml)	0.34	0.02

ρ: Spearman's correlation coefficient, BMI: body mass index.

In the recipients, plasma Ang2 measured at the first day after transplantation was associated with the length of hospital stay and IL-6 level (ρ 0.36, p=0.01 and ρ 0.34, p=0.02 respectively, table 3). In univariate lineair regression analysis, the first and second day after transplantation, plasma Ang2 correlated with plasma IL-6 (St. beta 0.33, p=0.02 and St. beta 0.43, p=0.002, respectively). No associations between plasma Ang2 and graft function were found.

Ang2 levels in the living kidney donor

Donor plasma Ang2 levels are shown in figure 1. In the living kidney donor, preoperative plasma Ang2 levels decreased significantly from 956 [510-768] pg/ml to 752 [461-661] pg/ml at organ retrieval (p=0.01). At 2 hours postoperatively, Ang2 levels increased significantly to 916 [535-844] pg/ml (p=0.002).

Ang2 levels during reperfusion

Arteriovenous Ang2 measurements over the reperfused kidney are shown in figure 2. Compared to the pretransplant Ang2 levels in the living kidney donor, an elevated trend in Ang2 levels was observed. No significant differences between arterial and venous plasma Ang2 were found.

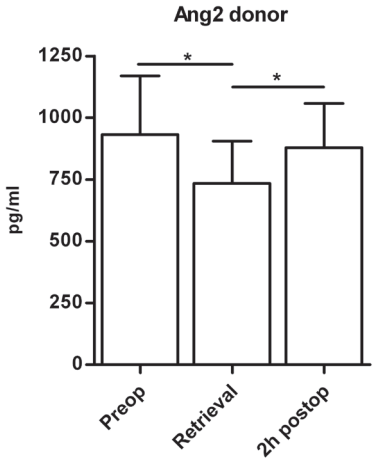

Figure 1. Preoperative, intra- and post transplantation systemic Ang2 plasma levels in living kidney donors. Error bars indicate mean±SEM of 53 living kidney donors. Preoperative plasma Ang2 levels decreased from 956 [510-768] pg/ml to 752 [461-661] pg/ml at organ retrieval (p=0.01). At 2 hours postoperatively, Ang2 levels increased to 916 [535-844] pg/ml (p=0.002).

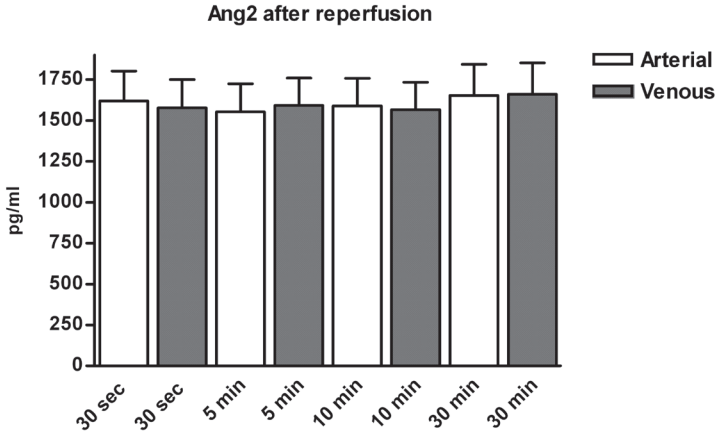

Figure 2. Arteriovenous Ang2 levels over the reperfused kidney. Error bars indicate mean±SEM of 53 arteriovenous Ang2 kidney measurements. No significant differences between arterial and venous plasma Ang2 were found.

Recipient Ang2 levels

Plasma Ang2 levels in the recipient are shown in figure 3. Ang2 levels decreased significantly from 2243 [723-3289] pg/ml preoperative to 1044 [556-1309] pg/ml 2 hours postoperative (p=0.003). No differences between plasma Ang2 one day after transplantation and Ang2 levels 2 hours after transplantation were determined.

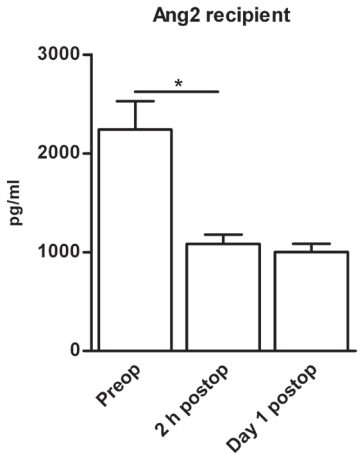

Figure 3. Systemic Ang2 plasma levels of 53 recipients after living kidney donor transplantation. Error bars indicate mean±SEM plasma Ang2 of 53 kidney recipients. Ang2 levels decreased from 2243 [723-3289] pg/ml preoperative to 1044 [556-1309] pg/ml 2 hours postoperative (p=0.003). No differences between plasma Ang2 one day after transplantation and Ang2 levels 2 hours after transplantation were found.

DISCUSSION

The current study is the first demonstrating systemic and local renal venous Ang2 levels throughout living donor renal transplantation. In both donor and recipient systemic Ang2 changes were observed while renal Ang2 release did not differ at different time points during the first 30 minutes of reperfusion.

In renal transplant recipients, Ang2 levels have been associated with renal function and all-cause mortality[26]. Although we did not find an association between systemic and/or renal Ang2 levels and transplantation outcome, these results are inconclusive due to the small study population and low incidence of events in our cohort.

The renal reperfusion Ang2 levels we found are not lower and show less variability than the study of de Vries and colleagues. Using the same technique they showed in six living and six deceased kidney donors, an increased Ang2 release shortly after reperfusion[21]. This is remarkable because of the relatively small study population compared to our study population. Furthermore, despite utilization of the same Ang2 ELISA the renal venous Ang2 levels we measured were in general considerably lower than the reperfusion levels determined by de Vries et al. Possibly, the lower Ang2 levels we measured during the donor and recipient procedure were caused by dilution. During both procedures patients received between 3000 and 5000 ml of crystalloids. Another explanation may be that less endothelial activation was already present and developed during nephrectomy in our healthy living donors, resulting in less WPB exocytosis of Ang2 during reperfusion. It has been well-known

that living donor kidney grafts are retrieved from healthy individuals selected on the absence of any disease, suffering from limited IRI and showing good function and high survival rate[30-33]. Therefore, the finding that in patients with normal kidney function after successful renal transplantation activation of the endothelial layer is indistinguishable from controls, underlines our speculation that endothelial activation in the living donors we studied may have been limited.

Without further studying endothelial activation of our donor-recipient couples, possibly via immunohistochemistry or real-time PCR of the retrieved renal biopsies, no definite conclusion in comparing the two living donor study populations can be made. This would be interesting since this previous study on local Ang2 release reports an increased local renal Ang2 plasma release in both living and deceased kidney donors and interestingly, no difference in Ang2 mRNA expression between living and deceased donor kidneys was observed[21]. This is remarkable as much evidence demonstrate a profound activation of the endothelium and inflammation in deceased donors, especially deceased brain dead donors, compared to living donors[3,34-36].

Since venous measurements of the reperfused graft during transplantation are an elegant method to study Ang2 release from the kidney itself and angiopoietins may reflect the immunogenic state and quality of the donor organ, future studies using this method are needed to draw more definite conclusions on the role of the Ang/Tie2-system in renal transplantation. That is, the Ang/Tie2-system has been shown to play a critical role in maintaining vascular stability while activated endothelium triggers an inflammatory response, affecting donor organ quality and function[37-41]. Ideally, circulatory and renal plasma Ang2 release together with the expression of Ang1 and Tie2 and the intensity of endothelial activation will be determined by immunohistochemistry and protein quantification in baseline living and deceased renal transplantation. This will possibly pave the way for performance enhancing intervention studies, targeting endothelial activation via the Ang/Tie2-system, possibly improving donor organ quality and subsequently, transplantation outcome.

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CHAPTER 4

Angiopoietin-2 associates with graft failure and mortality in renal transplant recipients

WH Westendorp
CA Keyzer
E van den Berg
C Krikke
H van Goor
SJL Bakker
HGD Leuvenink

Submitted

ABSTRACT

Background

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are involved in stabilizing vascular endothelium and may play a role in mortality and graft failure in renal transplant recipients (RTR). Early identification of RTR at risk could allow management, possibly via anti-Ang2 therapy. We aimed to investigate the association of Ang1 and Ang2 with graft failure and mortality in a prospective cohort of RTR. Elevated Ang2 levels have been demonstrated in sepsis which has pathophysiological similarities to the deceased brain dead donor. Therefore we also separately studied Ang2 associations in RTR transplanted with deceased donor kidneys.

Methods

Plasma Ang1 and Ang2 levels were measured in 552 RTR and 86 living kidney donors (LKD).

Results

Ang1 was higher in RTR than in LKD (p=0.002), while Ang2 was similar. For Ang1, no association with heart rate, Nt-pro-BNP or hsCRP was observed. Similar multivariate analysis demonstrated associations with Ang2 (all p<0.001). In deceased donor-RTR, Ang2 levels were higher compared to living donor-RTR. After adjustment for potential confounders, Ang2 levels were associated with graft failure (HR 3.64, 95%CI 1.17-11.28, p=0.03) and mortality (HR 1.53, 95%CI 1.03-2.27, p=0.04) after deceased donation.

Conclusions

Studies investigating the mechanism of the Ang/Tie2-system in renal transplantation are needed to provide more insight on cause and effect.

INTRODUCTION

The angiopoietin/Tie2 ligand-receptor system is involved in stabilizing the vascular endothelium and has been proposed as a potential therapeutic target in various conditions[1-3]. Angiopoietins are ligands that bind to the tyrosine kinase receptor Tie2, which is almost exclusively expressed by endothelial and hemopoietic stem cells[4]. Binding of Ang1 to the Tie-receptor leads to stabilization of the endothelium. In contrast, Ang2 destabilizes the blood vessels and enhances vascular leakage by priming the endothelial cells to respond to cytokines[5]. Ang1 is produced and immediately released at a constant rate by precursor platelets, pericytes and vascular smooth-muscle cells (SMCs) while Ang2 is stored in Weibel Palade bodies (WPB)[4,6,7]. The content of these endothelial-specific storage granules is rapidly released upon endothelial activation in response to, among others, thrombin, histamin and superoxide. Release of Ang2 leads to inflammation, coagulation and angiogenesis[4,5,8]. In the adult vasculature, a delicate balance of constitutive Ang1 expression and low-level Tie2 phosphorylation controls and maintains vascular quiescence, thus protecting the endothelium from excessive activation[9,10]. Ang1 and Ang2 therefore not only play important roles in the autocrine regulation of vascular stability and permeability, but also in the inflammatory balance.

Angiopoietin-2 (Ang2) is currently being evaluated as a promising biomarker in the field of acute pancreatitis[11]. Elevated Ang2 plasma levels have also been associated with a range of conditions such as myocardial infarction, trauma and sepsis[12,13]. Moreover, circulating Ang2 increases with the progression of chronic kidney disease (CKD), is predictive of mortality in CKD patients and correlates with severity of vascular disease in patients on dialysis[6,14,15]. Anti-Ang2 therapies have been studied in several preclinical models and anti-Ang2 phase III clinical trials that have been performed to date provided promising results[16-20].

For patients with end stage renal disease, renal transplantation has become the treatment of choice. However, graft failure remains an important problem and mortality rates remain high in RTR compared to the general population[21-27]. The origin of this high rate of mortality is multi-factorial with a high prevalence of a multitude of synergistically acting risk factors including morbidity, micro-inflammation and impaired graft function[24,26,28-31]. The involvement of endothelial dysfunction interacting with proteinuria in increasing the risk for mortality in RTR highlights the importance of vascular endothelium[32-34].

Ang2 release is triggered in human experimental endotoxemia and in sepsis elevated Ang2 is associated with increased mortality[35-37]. The known endotoxemia in DBD donors together with the pathophysiological similarities between sepsis and brain death makes the Ang/Tie2-system an interesting interventional target in DBD donors. Especially since endothelial activation has been shown to be a key factor in organs derived from DBD donors[38,39].

We aimed to investigate Ang1 and Ang2 levels among RTR and healthy controls. Additionally, we investigated associations of Ang1 and Ang2 with clinical parameters in RTR. We moreover hypothesized that Ang1 and Ang2 are associated with the occurrence of graft failure and mortality in stable, outpatient RTR. Because of the suggested role of Ang2 in endothelial activation in deceased donor kidneys, secondary analyses were performed in recipients of a kidney derived from deceased donors.

MATERIALS AND METHODS

Study populations

For the current analysis, we used plasma of a prospective observational single-center cohort study in which all RTR (≥ 18years) with a functioning graft, who visited our outpatient clinic between 2008 and 2010, were invited to participate[40]. A total of 707 out of 817 eligible RTR (87%) signed written informed consent. Plasma Ang1 and Ang2 levels were measured in 552 RTR (78.1%). As a healthy control group, we included 86 subjects after they signed written informed consent, which were evaluated and approved for living kidney donation in our center. None had a history of kidney disease or diabetes mellitus. Hypertension, if present, was treated with a maximum of three antihypertensive drugs. The Institutional Review Board approved the study protocol (METc 2008/186), which was in adherence to the Declaration of Helsinki.

Urine and plasma parameters

All participants were carefully instructed to collect a 24-hour urine sample according to a strict protocol at the day prior to their visit to the outpatient clinic. Urine was collected with chlorhexidin was added as antiseptic agent. Blood was drawn in the morning after completion of the 24h urine collection. For the control group, the 24h urine collection was performed before donor nephrectomy. Plasma Ang1 and Ang2 levels were measured via enzyme-linked immunosorbent assay (ELISA) Duosets (R&D Systems, Minneapolis, USA).

Plasma and urinary concentrations of albumin, HbA1c, hsCRP, Nt-pro-BNP and total protein levels were measured using a Roche Modular chemistry analyzer (Roche Diagnostics, USA).

Renal function was assessed by estimating Glomerular Filtration Rate according to the Chronic Kidney Disease Epidemiology Collaboration (eGFR). Serum creatinine was determined using an enzymatic essay on a Roche Modular analyzer.

Clinical parameters

All measurements were performed during a morning visit to the outpatient clinic after an 8-12hr overnight fasting period. Blood pressure (mmHg) of RTR and living

donors was measured according to a strict protocol as previously described[41]. Participants were in half-sitting position while systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate were measured with a semi-automatic device (Dinamapâ 1846, Critikon, Tampa, FL, USA). Measurements were performed every minute for fifteen minutes and the last three values were averaged. Information on participants' health status, medical history and medication use was obtained from patient records. Information about the renal transplantation was extracted from the local University Medical Center Groningen renal transplantation database. Body weight and height were measured with participants wearing indoor clothing without shoes. BMI was calculated as weight divided by height squared (kg/m^2) and Body Surface Area (BSA) was estimated applying the universally adopted formula of Dubois and Dubois[42].

Clinical endpoints

The primary endpoints of this study were mortality and death-censored graft failure defined as return to dialysis or re-transplantation. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status and cause of death. General practitioners or referring nephrologists were contacted in case the status of a patient was unknown. Endpoints were recorded until the end of May 2013. There was no loss to follow-up.

Statistical analyses

Data are presented as mean ± SD (standard deviation), median (range) or [interquartile range] (IQR) and number (percentage) for normally, non-normally distributed data, and nominal data, respectively. Analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Normality was tested with the Kolmogorov-Smirnov test. Skewed data were normalized for analyses by natural-logarithm transformation (LN) transformation. In all analyses, a two-sided *P*-value of less then 0.05 was considered to indicate statistical significance. Differences between RTR and healthy controls, and deceased donor- RTR vs. living donor-RTR, were tested with the t-test for independent samples or the Mann Whitney U test in case of continuous variables and the Chi-square test in case of dichotomous variables. Age and sex-adjusted associations of Ang1 or Ang2 levels with various clinical parameters were analyzed with linear regression analysis (model 1). Further adjustments were made for eGFR and transplantation vintage (model 2), use of antihypertensives (model 3) and additional adjustment for either Ang1 or Ang2 (model 4).

Regression coefficients are given as standardized betas. In prospective analyses, we investigated associations of plasma Ang1 and Ang2 with death-censored graft failure and all-cause mortality in RTR. Subsequently, associations of plasma Ang1 and Ang2 with graft failure and mortality were studied separately in RTR with a graft retrieved from a deceased or living donor. Kaplan-Meier survival curves

and logrank tests were performed between groups to asses the difference in death-censored graft survival or patient survival rates. We performed crude Cox regression analyses for the association with death-censored graft failure (model 1). Additionally analyses were adjusted for age, sex and donor type (model 2), cold ischemia time, number of transplantations, number of mismatches (model 3), and years since transplantation, albuminuria (mg/24h) and renal function (eGFR) (model 4). For the association with all-cause mortality, we first performed crude Cox regression analyses (model 1) and adjusted for age and sex (model 2), and additionally adjusted for diabetes and donor type (model 3), and additionally adjusted for years since transplantation and renal function (eGFR) (model 4). Possible interactions between confounders were tested.

RESULTS

RTR vs. controls

The characteristics of RTR and controls are shown in table 1. Baseline measurements in RTR were performed at a median of 5.4 [IQR 1.9-12.2] years after kidney transplantation. RTR and controls were similar with respect to age, sex, BMI and BSA. Plasma Ang1 concentration was higher in RTR; 1715 (range 134-9776) vs. 1207 (151-5638) in controls, p=0.002, but Ang2 concentration did not differ between both groups (607 (0.12-2467) in controls vs. 665 (0.96-9242) in RTR; p=0.14). As anticipated, serum creatinine and eGFR were significantly worse in RTR than in healthy subjects (both p<0.001). This was accompanied by higher blood pressure values in RTR than in controls (both p<0.001). Compared to controls, RTR had significantly higher levels of albumin excretion, glycosylated haemoglobin (HbA1c) and Nt-pro-BNP (all p<0.001). Of the 552 RTR, 355 recipients received a kidney from a deceased donor, 197 recipients received a kidney from a living donor. A comparison of RTR according to donor type is also presented in table 1. Plasma Ang1 concentration did not differ between these groups (p=0.96). Ang2 levels were higher among RTR of a deceased donor kidney (p=0.003). Deceased donor-RTR had significantly higher levels of Nt-pro-BNP compared to living donor-RTR (p<0.001).

Independent associations of Ang1 and Ang2 levels with clinical parameters in RTR

Age and sex-adjusted regression coefficients for the associations of plasma Ang1 and Ang2 with cardiovascular parameters are presented in table 2 (model 1). After adjustment for potential confounders (model 2), plasma Ang1 was significantly associated with DBP (β=0.07; p=0.04). Further adjustment for potential confounders did not change the significant associations of Ang2 with heart rate (β=0.12; p=0.001), Nt-pro-BNP (β=0.12; p<0.001) and hsCRP (β=0.17; p<0.001). The association of Ang2 with HbA1c was lost after adjustment for the use of antihypertensives (model 4).

Table 1. Baseline characteristics of 86 healthy controls and 552 renal transplant recipients at the day of their visit to the outpatient clinic

Characteristics	Healthy controls n=86	RTR n=552	p	RTR of DD n=352	RTR of LD n=200	p
Plasma Ang1 (pg/ml)	1207 [151-5638]	1715 [134-9776]	0.002	1738 [141-9601]	1656 [134-9776]	0.471
Plasma Ang2 (pg/ml)	607 [0.12-2467]	665 [0.96-9242]	0.141	721 [5-9242]	591 [1-6860]	0.003
Demographics						
Age (y)	43 ± 16	53 ± 13	0.417	55 ± 12	49 ± 13	<0.001
Male sex, n (%)	50	54.3	0.45	64	36	0.61
BMI (kg/m^2)	27 ± 3.5	27 ± 4.7	0.399	27 ± 5	26 ± 4	0.208
BSA (m^2)	1.97 ± 0.2	1.93 ± 0.2	0.178	1.94 ± 0.2	1.95 ± 0.2	0.320
Medication use						
Antihypertensive (%)	16.3	87.7	<0.001	90	85	0.094
Statins (%)	3.5	52.4	<0.001	54	51	0.736
Hemodynamic parameters						
SBP (mmHg)	125 ± 15	136 ± 18	<0.001	136 ± 18	136 ± 16	0.911
DBP (mmHg)	76 ± 10	82 ± 11	<0.001	82 ± 11	84 ± 11	0.005
MAP (mmHg)	97 ± 18	107 ± 15	<0.001	107 ± 15	108 ± 15	0.277
Heart rate (bpm)	66 ± 9	69 ± 12	0.068	68 ± 12	69 ± 11	0.026
Renal function parameters						
eGFR (ml/min)	90 ± 17	50 ± 18	<0.001	48 ± 19	51 ± 18	0.080
Serum creatinine (μmol/L)	73 ± 13	134 ± 51	<0.001	140 ± 60	135 ± 58	0.270
Urinary protein (gram/24h)	0.06 ± 0.1	0.36 ± 0.75	<0.001	0.45 ± 0.9	0.33 ± 0.7	0.010
Albumin excretion (mg/L)	3.3 ± 5.3	99 ± 257	<0.001	124 ± 292	107 ± 306	0.026
Serum parameters						
HbA1c (%)	5.5 ± 0.3	6 ± 0.8	<0.001	6 ± 0.8	6 ± 0.9	0.123
hsCRP (mg/L)	2.9 ± 7.6	4.3 ± 9.2	0.072	4.8 ± 9.4	3.8 ± 8.9	0.179
Nt-pro-BNP (ng/L)	56 ± 58	1018 ± 5414	<0.001	1458 ± 6398	382 ± 630	<0.001

Data are presented as mean ± SD, % or median [range]. Abbreviations: RTR: renal transplant recipients; BMI, body mass index; BSA: body surface area; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; eGFR: estimated Glomerular Filtration Rate according to the Chronic Kidney Disease Epidemiology Collaboration; HbA1c: glycosylated hemoglobuline; hsCRP: high-sensitive C-Reactive Protein; Nt-pro-BNP: N-terminal pro-Brain Natriuretic Peptide. P for difference was tested by the Mann-Whitney U test for continuous variables or Chi-square test for binary variables.

Table 2. Regression coefficients for the association of plasma Ang1 and Ang2 with clinical parameters in 552 renal transplant recipients

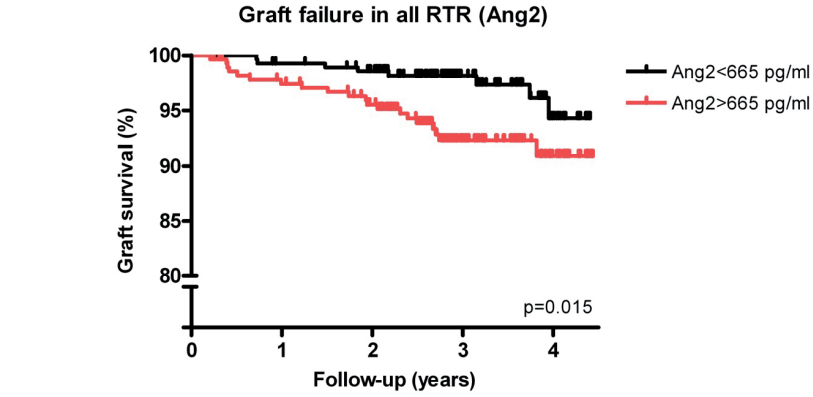
	Model 1		Model 2		Model 3		Model 4	
Dependent variable	β	p	β	p	β	p	β	p
Angiopoietin-1*								
SBP (mmHg)	0.04	0.27	0.05	0.20	0.04	0.23	0.04	0.28
DBP (mmHg)	0.07	0.04	0.07	0.04	0.07	0.05	0.07	0.09
MAP (mmHg)	0.04	0.26	0.05	0.19	0.05	0.21	0.05	0.25
Pulse pressure (mmHg)	-0.005	0.90	<0.001	0.99	-0.002	0.95	<0.001	0.99
Heart rate (bpm)	0.04	0.29	0.04	0.35	0.04	0.32	-0.008	0.84
Nt-pro-BNP* (ng/L)	0.01	0.78	0.03	0.27	0.03	0.32	-0.01	0.71
hsCRP* (mg/L)	0.04	0.34	0.04	0.28	0.04	0.30	-0.02	0.61
HbA1c* (%)	0.05	0.18	0.04	0.23	0.04	0.27	0.02	0.70
Angiopoietin-2*								
SBP (mmHg)	0.02	0.53	0.02	0.52	0.02	0.59	0.01	0.90
DBP (mmHg)	0.04	0.27	0.04	0.26	0.04	0.29	0.02	0.69
MAP (mmHg)	0.02	0.57	0.02	0.58	0.02	0.64	0.002	0.97
Pulse pressure (mmHg)	-0.003	0.94	-0.003	0.92	-0.007	0.85	-0.007	0.86
Heart rate (bpm)	0.12	0.001	0.12	0.001	0.13	0.001	0.13	0.001
Nt-pro-BNP* (ng/L)	0.14	<0.001	0.12	<0.001	0.12	<0.001	0.12	<0.001
hsCRP* (mg/L)	0.17	<0.001	0.16	<0.001	0.16	<0.001	0.17	<0.001
HbA1c* (%)	0.08	0.04	0.08	0.03	0.08	0.04	0.07	0.07

Coefficients are provided as standardized betas, referring to the number of standard deviations the dependent variable changes, per standard deviation increase of Ang1/Ang2.
Model 1: adjusted for age, sex
Model 2: additionally adjusted for eGFR and years since transplantation
Model 3: additionally adjusted for use of antihypertensives
Model 4: additionally adjusted for either Ang1 or Ang2
*Natural-logarithm transformation (LN) for analyses. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; hsCRP, high-sensitive CRP; HbA1C, glycosylated hemoglobuline; eGFR, estimated glomerular filtration rate according to the Chronic Kidney Disease Epidemiology Collaboration

Association of Ang1 and Ang2 with death-censored graft failure

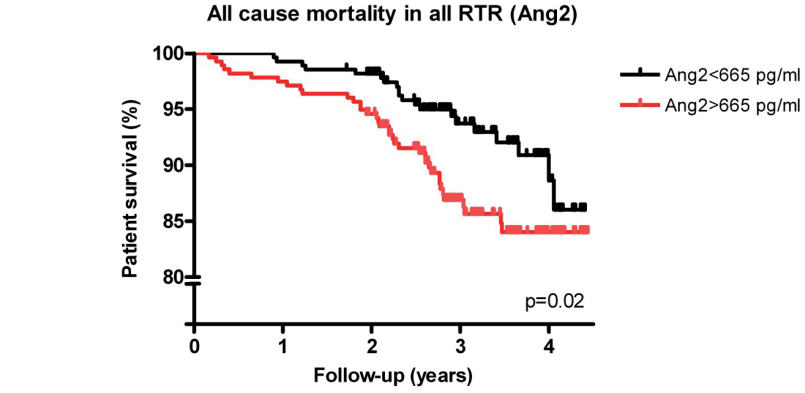
Median follow-up from baseline was 3.1 [IQR 2.6-3.8] years. During this follow-up 28 (5%) of 552 RTR developed graft failure. For Ang1, no association with graft failure was observed (Log-rank test p=0.17). Kaplan-Meier curves for death-censored graft failure stratified into Ang2 levels under vs. above the median (665 pg/ml) are shown in figure 1. Incidence of graft failure during follow-up in the low Ang2-group was 8 out of 276 (3%) vs. 20 out of 276 (7%) in the high Ang2-group. Ang2 levels above the median were significantly associated with death-censored graft failure (Log-rank test p=0.015). The association between plasma Ang1 and Ang2

levels with graft failure was assessed using Cox regression analysis (table 3). After adjustment for age and sex, the significant association between Ang2 levels and graft failure was lost.



No. at risk					
Ang2<665 pg/ml	276	273	262	133	38
Ang2>665 pg/ml	276	263	247	137	47

Figure 1. Kaplan-Meier curves and numbers at risk for death-censored graft survival of 552 renal transplant recipients (RTR). Survival rates stratified into high and low Ang2 level, under versus above the median of 665 pg/ml. Log-rank test p=0.015.



No. at risk					
Ang2<665 pg/ml	275	274	263	133	40
Ang2>665 pg/ml	277	271	260	149	51

Figure 2. Kaplan-Meier curves and numbers at risk for patient survival of 552 renal transplant recipients (RTR). Stratified into high and low Ang2 level, under versus above the median of 665pg/ml. Log-rank test p=0.02.

Table 3. Cox regression analyses for prediction of death-censored graft failure based on plasma Ang1 and Ang2 levels in 552 renal transplant recipients

	Angiopoietin-1*			Angiopoietin-2*		
	N_{total}= 552/ N_{events}= 28					
	HR	95% CI	p	HR	95% CI	p
Model 1	1.45	0.63-3.30	0.38	1.75	1.00-3.07	0.049
Model 2	1.32	0.57-3.05	0.51	1.71	0.95-3.08	0.07
Model 3	1.38	0.60-3.15	0.45	1.79	0.98-3.23	0.05
Model 4	1.70	0.56-5.19	0.35	2.02	0.97-4.23	0.06

*Angiopoietin-1 and angiopoietin-2 were natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and donor type
Model 3: model 2 plus adjustment for cold ischemia time, number of transplantations, number of mismatches
Model 4: model 3 plus adjustment for transplantation vintage, albuminuria and renal function (eGFR, CKD)

Association of Ang1 and Ang2 with all-cause mortality

During follow-up 59 (11%) of 552 RTR died. Kaplan-Meier curves for all-cause mortality stratified into Ang2 levels under vs. above the median (665 pg/ml) are shown in figure 2. For Ang1, no association with mortality was observed (Log-rank test p=0.46). Incidence of mortality during follow-up in the low Ang2-group was 22 out of 276 (8%) vs. 37 out of 276 (13%) in the high Ang2-group. Ang2 levels above the median were significantly associated with mortality (Log-rank test p=0.02). The association between plasma Ang1 and Ang2 levels with all-cause mortality was assessed in table 4 using Cox regression analysis. After adjustment for all possible confounders (model 4), Ang2 levels were significantly associated with mortality (p=0.045).

Secondary analysis with Ang2 according to donor type

In analyses stratified according to donor type, a difference in associations of plasma Ang2 with graft failure was observed (table 5). After adjustment for age, sex, cold ischemia time, number of transplantations, number of mismatches, years since transplantation, albuminuria and renal function (model 4), plasma Ang2 levels were significantly associated with graft failure in deceased donor-RTR (p=0.03). No association between plasma Ang1 and graft failure in the separate donor types was found. For all-cause mortality as well, a difference in associations of plasma Ang2 was observed in stratified analysis according to donor type (table 6). After adjustment for age, sex, diabetes, donor type, years since transplantation and renal function (model 4), plasma Ang2 levels were significantly associated with mortality in deceased donor-RTR (p=0.04). No association between plasma Ang1 and all-cause mortality in the separate donor types was found.

Table 4. Cox regression analyses for prediction of all-cause mortality based on plasma Ang1 and Ang2 levels in 552 renal transplant recipients

	Angiopoietin-1*			Angiopoietin-2*		
	N_{total}= 542/ N_{events}= 59					
	HR	95% CI	p	HR	95% CI	p
Model 1	1.03	0.61-1.74	0.90	1.73	1.16-2.58	0.007
Model 2	1.13	0.65-1.96	0.68	1.76	1.18-2.62	0.005
Model 3	1.09	0.64-1.86	0.76	1.52	1.05-2.21	0.03
Model 4	1.08	0.62-1.89	0.78	1.46	1.01-2.10	0.045

*Angiopoietin-1 and angiopoietin-2 were natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age and sex
Model 3: model 2 plus adjustment for donor type and diabetes
Model 4: model 3 plus adjustment for transplantation vintage and renal function (eGFR, CKD)

Table 5. Cox regression analyses for prediction of death-censored graft failure based on plasma Ang2 levels in 552 renal transplant recipients separated per donor type

	Angiopoietin-2*					
	Deceased donor			Living donor		
	N_{total}= 352/ N_{events} = 20			N_{total}= 200/ N_{events}= 8		
	HR	95% CI	p	HR	95% CI	p
Model 1	2.36	1.15-4.84	0.02	1.03	0.50-2.14	0.94
Model 2	2.38	1.10-5.13	0.03	1.13	0.54-2.39	0.75
Model 3	2.82	1.23-6.47	0.02	1.16	0.47-2.83	0.75
Model 4	3.64	1.17-11.28	0.03	0.98	0.34-2.81	0.96

*Angiopoietin-2 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. HR: Hazard Ratio; CI: Confidence Interval.
Model 1: crude model
Model 2: adjusted for age, sex
Model 3: model 2 plus adjustment for cold ischemia time, number of transplantations, number of mismatches
Model 4: model 3 plus adjustment for transplantation vintage, albuminuria and renal function (eGFR, CKD)

DISCUSSION

The major finding of this study is that Ang2 plasma levels are associated with death-censored graft failure and all-cause mortality after renal transplantation in a large cohort of kidney transplant recipients. The association between Ang2 levels and graft failure and mortality is independent of established risk factors. We additionally found higher plasma Ang2 levels in RTR who received a kidney from a deceased donor compared to those from RTR who received a kidney from a living

Table 6. Cox regression analyses for prediction of all-cause mortality based on plasma Ang2 levels in 552 renal transplant recipients separated per donor type

	Angiopoietin-2*					
	Deceased donor			Living donor		
	N_{total}= 353/ N_{events} = 50			N_{total}= 189/ N_{events} = 9		
	HR	95% CI	p	HR	95% CI	p
Model 1	1.75	1.15-2.66	0.009	1.08	0.45-2.60	0.86
Model 2	1.78	1.18-2.70	0.007	1.07	0.44-2.61	0.88
Model 3	1.61	1.08-2.41	0.02	0.96	0.39-2.39	0.93
Model 4	1.53	1.03-2.27	0.04	1.04	0.36-3.01	0.95

*Angiopoietin-2 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age and sex
Model 3: model 2 plus adjustment for diabetes
Model 4: model 3 plus adjustment for transplantation vintage and renal function (eGFR, CKD)

donor. In deceased donor-RTR, secondary analysis demonstrated an association of plasma Ang2 with both graft failure and mortality.

Our findings extend previous data in which Ang2 levels predicted mortality in CKD patients and critically ill patients[24,31]. To further underline the clinical importance of Ang2 in renal transplantation and different donor types, we here demonstrate its predictive value after renal transplantation in a large cohort of renal transplant recipients. The significance of increased Ang2 is also reflected by the identification of Ang2 as a promising biomarker in predicting severe acute pancreatitis[43] and associations between Ang2 and critical illness such as acute respiratory distress syndrome, myocardial infarction, sepsis and trauma[12]. To clarify the potential of clinically measuring Ang2 in the scope of CVD and mortality after renal transplantation, it should critically be compared to other clinical signs of CVD or predictors of mortality in future prospective studies to see if the Ang2 levels rise prior to the established signs.

Since deceased donors suffer from unfavorable inflammatory responses which cause endothelial activation, we compared the Ang1 and Ang2 levels between deceased donor-RTR and living donor-RTR. As anticipated, deceased donor-RTR have higher Ang2 levels compared to living donor-RTR. Whereas the Ang2 levels we found in the control and living donor-RTR group are quite similar to the levels measured in other healthy individuals[44].

Although early after transplantation a rapid Ang2 release has been observed by others, we did not find a difference in Ang2 levels between the whole RTR group and controls, possibly due to the longer time frame between transplantation and sample withdrawal in our study groups[45]. It is possible that the elevated Ang2 levels

we found in the deceased donor-RTR are a consequence of pre-existent damage caused during time of donation[39,45,46].

At this period of time after transplantation, the difference in vascular status between the RTR and controls may not be reflected by the rapidly responding Ang2 or hsCRP levels but possibly by the constant high expression of Ang1 which correlates to the magnitude of vascular resistance[47,48]. Our results show that the increased mortality risk among RTR is independent of Ang1 levels, supporting the hypothesis that Ang2 is the more dynamic player, reacting to donor type, of these two growth factors.

The difference in mortality we found for Ang2 levels under vs. above the median in Kaplan-Meier analyses of mortality and graft failure was further extended by Cox regression analyses, in which we adjusted for potential confounders. The crude association remained after these adjustments. These results are in line with those of a case control study, in which it was reported that serum Ang2 levels predict mortality in kidney transplant recipients[49].

Our findings point to an unfavorable role for Ang2 in the development and/or progression of a detrimental cardiovascular profile following renal transplantation. We found significant independent relationships of Ang2 with hsCRP and Nt-pro-BNP in our multivariate lineair regression analysis. This is similar to previous data in which Ang2 levels are positively associated with CRP in another cohort of renal transplant recipients[49]. Similar findings were done at the onset of COPD exacerbations[49,50]. Moreover, Ang2 levels are positively associated with hsCRP in patients with coronary heart disease[50,51].

A potential mechanistic explanation for our findings is that Ang2 is involved in the pathogenesis of vascular inflammation, endothelial activation and atherosclerosis. Plasma Ang2 expression is increased in atherosclerotic plaques and correlates with plaque microvascular density and matrix metalloproteinase 2 (MMP-2) activity[52]. Ang2 has also been identified as a link between kidney fibrosis and arterial stiffness since blockade attenuated expression of monocyte chemokines, profibrotic cytokines and collagen in aorta of mice after 5/6 subtotal nephrectomy[53]. Although the expression of Ang2 has been known to be tightly controlled and is strongly increased following stimulation by cytokines, growth factors and environmental factors, the mode of Ang2 secretion by WPB has never been established[2,4,54]. However, there is evidence that this process is inhibited by the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway, which plays an important role in vascular growth and stabilization[55]. The physiological basis for the association between elevated circulating Ang2 and graft failure remains to be unraveled. In acute settings like pancreatitis, angiopoietins play a role in the first hour of the inflammation process, whereas their long-term effects are associated with vascular remodeling[44]. Even though the exact mechanism has not yet been completely elucidated, the angiopoietin/Tie2 ligand-receptor

system seems to have a distinct regulatory function in acute inflammation and structural remodelling. The resting endothelium expresses Ang2 weakly and stores Ang2 in WPB from where it can be made available quickly following stimulation, suggesting a role of Ang2 in controlling rapid vascular adaptive processes[4]. In adult mice and human, Ang2 is only expressed at sites of vascular remodelling[2]. Possibly RTR bear a pre-inflammatory state which aggravates in case there is a risk for organ failure, reflected by increased Ang2-supported endothelial activation[12,56]. Many studies have focused on anti-Ang2 therapy in the treatment of chronic rejection in rat cardiac allografts, malignant tumors including monoclonal antibodies and siRNAs[18-20,57,58]. None of them is evaluated in the setting of renal transplantation, although targeting Ang2 to attenuate inflammation may provide a novel therapy for graft failure and patient mortality in RTR, especially in the setting of deceased donation.

Several limitations of our study deserve acknowledgement. This study is an observational epidemiologic study, which makes it difficult to draw conclusions on causality. In general, statistical significance in observational studies suggests, but does not confirm, biologic significance. Whether the significant relation between Ang2 and mortality in RTR is a causal or an associative relation remains to be determined. However, the association between Ang2 and mortality and graft failure remained significant in multivariate Cox regression analyses, including renal function, suggesting that this association is independent of confounding factors. In addition, our study population was enrolled from a single center and was predominantly Caucasian which limits the generalizability of this study. Second, specific indices of vascular inflammation and potential subclinical atherosclerosis, as intermediate endpoints, were not assessed in this cohort. The Ang1 concentration we measured might be explained by variation in sample processing. Unlike Ang2, Ang1 is not exclusively expressed by the vascular wall. A high amount of Ang1 is also found in platelets[59]. False positive Ang1 levels can therefore result from the *ex vivo* activation of platelets in serum tubes[7]. Another possibility for the increased Ang1 levels could be the use of immunosuppressive treatment, most probably steroid treatment[60].

It is an observational study of well defined RTR with a large sample size and complete follow-up. Extensive data collection, including data from 24h urine samples allowed for adjustment for many potential confounders in our analysis. We speculate that targeting the Ang/Tie2-system, more specifically Ang2, may be a strategy to reduce the observed detrimental associations of Ang2 with patient and graft survival, especially after deceased donation. However, more studies investigating the cause of Ang2 release and its effects are needed to clarify the role of the angiopoietin/Tie2-system in renal transplantation.

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CHAPTER 5

Lymphatic vessels: an emerging participant in the pathology of pre-transplant kidney biopsies?

WH Westendorp*
S Yazdani*
H Tent
MCRF van Dijk
MLC Bulthuis
HS Hofker
GJ Navis
RJ Ploeg
JL Hillebrands
J van den Born
H van Goor
HGD Leuvenink

*These authors contributed equally

Submitted

ABSTRACT

Organs derived from deceased brain dead (DBD) donors show worse function than those from living donors (LD), possibly due to the inflammatory burst in DBD donors. Recently, renal lymphangiogenesis has been documented as an important player in inflammation, fibrosis and tubulointerstitial remodeling. We studied lymphatic vessels (LVs) in pre-existent renal damage in kidney biopsies from DBD and LD and investigated their associations with other histological parameters. In biopsies of 73 DBD and 131 LD, the degree of focal glomerulosclerosis (FGS), interstitial fibrosis (IF), intima thickness and vascular hyalinosis were analyzed. Sections were evaluated for number of macrophages, granulocytes, lymphatic vessel density (LVD), α-SMA and interstitial fibrosis (IF). Arteriopathy, tubulointerstitial inflammation and fibrosis were increased in DBD compared to LD ($p<0.05$). LVs were mainly localized in the adventitial area around arteries and arterioles. LVD was ~1.7-fold higher in DBD kidneys compared to LD kidneys ($p<0.001$). Within the DBD group, LVD and other histological parameters were significantly higher in donors that died as a result of a CVA compared to traumatic BD. The increased signs of arteriopathy, IF and inflammation in DBD donor biopsies correlated to renal LVD which stresses the importance of investigating LVD in prospective studies on kidney transplant biopsies.

INTRODUCTION

The majority of organs for transplantation are derived from deceased brain dead (DBD) donors. Unfortunately, the process of brain death is associated with a cascade of hemodynamic, inflammatory, hormonal and immunologic events that negatively affect function and outcome of transplanted kidneys[1-3]. In kidney transplantation, Living Donation (LD) is a well-established way to increase the number of transplants and in many countries this is common practice. Transplantation outcome of LD kidneys is superior compared to DBD kidneys[4]. The critical importance of donor organ quality, ability to withstand transplant-related injury, and capacity for repair in determining short- and long-term outcome is well recognized. In the kidney, minor interstitial, vascular and glomerular damage can already be present without clinical signs of deterioration such as proteinuria or decreased kidney function due to the large reserve capacity. Besides clinical parameters, donor baseline biopsies have been used to assess the quality of a donor organ mainly in centers in the USA[5-8]. One of the components of the inflammatory events that take place in the DBD donor is the influx of macrophages and granulocytes[9-11]. This infiltration is initially meant to modulate the inflammatory process and subsequently take part in repairing process once the initial trigger has vanished. Ultimately the continuing presence of inflammation becomes pathological, resulting in renal fibrosis with damaging consequences for the donor organ[12]. Several studies have evaluated histological lesions in assessing organ quality as well as predicting early- and long-term transplantation outcomes[13-20]. Interstitial fibrosis (IF), fibrous intimal thickening, focal glomerulosclerosis (FGS) and arteriolar hyalinosis have variably been identified as parameters in predicting delayed graft function (DGF) and/or poorer graft function and survival[7,14,21-23].

Lymphatic vessels (LVs) and its outgrowths (lymphangiogenesis) seem to play an important role in kidney pathophysiology, especially in renal transplantation[24,25]. However, conflicting results on the role of LVs and lymphangiogenesis in organ transplantation have been reported. Lymph vessel density (LVD) has been reported to increase early after renal transplantation independently of signs of rejection, and associates with different kinds of inflammatory reactions[25,26]. Pre-existing lymphatic vessels in corneal transplant recipients significantly reduced corneal graft survival; while recipients' pre-transplant blocking of lymphangiogenesis prolonged this graft survival[27,28]. The exact role of LVs within pre-existing renal damage at time of kidney donation and transplantation has not been established yet.

Baseline histologic lesions at time of transplantation and their effect on kidney function and graft survival have been evaluated using different scoring systems. Although lymphangiogenesis has been proposed to be an interesting novel therapeutic target in kidney transplantation[25], none of these studies[5,15,18,19,29-32] have evaluated LVD and its association with other histological changes in biopsies from (pre)transplant DBD and living donors.

In the light of the current knowledge about kidney transplant biopsies, we aimed to study our single center kidney transplant population. Therefore we quantified LVD and other pre-existent damage parameters in kidney biopsies of DBD and living donors, and investigated the association between these structural changes. We hypothesize that LVD, pre-fibrotic, fibrotic and inflammatory glomerular, vascular and interstitial changes are increased in biopsies of DBD donors compared to biopsies of LD. Next, we expect the origin of brain death to be reflected by these pre-existent renal damage parameters. To provide more insight in the development of pre-existent renal damage in the DBD donor, we performed secondary analyses comparing all parameters between DBD donors who died due to a cardiovascular accident (CVA) with DBD donors who died because of a traumatic accident.

METHODS

Biopsies from 238 donor kidneys were consecutively collected during organ procurement and transplantation procedures from 2005 through 2008 at the University Medical Center Groningen. Renal biopsies were obtained from DBD (n=73) and living donors (n=131) at three different time points: just prior to donation (before kidney recovery and start of preservation), at the end of cold ischemia and 45 min after reperfusion. All DBD donors were declared brain dead on the intensive care and samples were collected after declaration of brain death with consent from the legally authorized relative according to the Dutch Transplantation Law. According to this law, general consent for organ donation and transplantation includes consent for research projects. Nevertheless, the Institutional Review Board approved the study protocol, which was in adherence to the Declaration of Helsinki and all methods were carried out in accordance with the approved guidelines.
All clinical data were anonymized prior to analysis. Biopsies were taken using a 16-gauge needle (Acecut®, TSK Laboratory, Japan), and subsequently stored in formalin and paraffin fixed until analysis. Renal donor biopsies from 21 donors showed merely non-cortical tissue and were excluded from analysis. Since three biopsies were taken from each kidney the exclusion was not considered to have any effect on the outcome. Screening or follow-up data from 13 donors were missing. This results in a total of 204 donors eligible for analysis.

Immunohistochemistry

Deparaffinized sections were subjected to heat-induced antigen retrieval either by overnight incubation in a 0.1 M Tris-HCl buffer (PH 9.0) at 80°C, or 10 mM Tris-1 mM EDTA buffer (PH 9.0) for 15 minutes in the microwave. Endogenous peroxidase was blocked with 0.3% H2O2 in PBS for 30 min. Sections were incubated with D2-40 antibody (clone D2-40, diluted 1:40, DAKO, Glosstrup, Denmark) for lymphatic vessels, α-SMA antibody (clone1A4, diluted 1:10000, Sigma, Zwijndrecht, The

Netherlands), CD68 antibody (clone PGM-1, diluted 1:250, DAKO, Glosstrup, Denmark) for macrophages, or with monoclonal antibody 12.8[33] (clone 12.8, diluted 1:10) for 60 min at room temperature for neutrophils. This monoclonal antibody was a gift from Prof. dr. C.A. Stegeman (University Medical Center Groningen, the Netherlands). Binding of the antibody was detected using sequential incubations (30 min each) with PO-labeled rabbit-anti-mouse (RAMPO, diluted 1:100, DAKO) and PO-labeled goat-anti-rabbit antibodies (GARPO, diluted 1:100, DAKO). PO activity was developed using 3, 3'-diaminobenzidine tetrachloride (DAB) for 10 min. Sections stained for lymphatic vessels and macrophages were counterstained with PAS. Sections for neutrophilic granulocytes were counterstained with hematoxylin. Interstitial cortical lymphatic vessels were counted manually (Image J version 1.46r) and expressed as the number of LVs per mm^2 of cortical area, lymph vessel density (LVD). As we do not expect any new lymph vessel formation during cold ischemia or after reperfusion, the mean score of all counted lymphatic vessels in three biopsy time points were calculated and used for statistical analysis. α-SMA expression was determined by Positive Pixel Count (Aperio Imagescope version 10.2.2). Macrophages and neutrophilic granulocytes were counted manually in the interstitium. Since the influx of inflammatory cells does not occur during cold ischemia, we used the scores from the second biopsy for analysis. Glomeruli, the renal medulla and vessels were excluded from counting and surface area calculation. For α-SMA expression analysis, average score from all three biopsy time points were used. All scores were adjusted for biopsy surface area (BSA).

Morphological damage

To evaluate the degree of FGS, IF and vascular hyalinosis, paraffin sections (3 μm) were stained with Periodic Acid Schiff (PAS) and scored by a renal pathologist blinded for donor type and other clinical characteristics. A surface calculation using Aperio Imagescope was performed on all the sections. IF was defined as expansion of the interstitial space, with or without the presence of atrophied and dilated tubules and thickened tubular basement membranes. The glomeruli, renal papilla and vessels were excluded from the calculated fibrotic areas. The degree of IF was scored on a scale of 5: 0%, 0-10%, 10-25% 25-50%, 50-75% and 75-100% of biopsy surface area. FGS was scored as % of total glomeruli. Vascular hyalinosis was scored as none, scarce, moderate, or severe. A mean score was calculated from the three different time points and adjusted for surface area. To calculate a mean score, at least three arteries had to be present.

Arterial intima thickness

Intima surface was expressed as a percentage of the media surface, to provide a standardized estimation of the severity of intima thickening independent of vessel size. A higher percentage represents a relatively thicker intima. For this purpose, all

arteries present in the biopsies were analyzed in the α-SMA stained sections. For each vessel, media and intima were outlined to calculate individual surfaces using Aperio Imagescope. Intima surface was expressed as percentage of media surface. For each donor, the separate vessel scores of all three biopsy time points were mediated. At least values from three vessels were required to calculate an overall score.

Donor kidney function measurements

Through constant low-dose infusion of the radiolabeled tracer ^{125}I-iothalamate the glomerular filtration rate (GFR) was measured in the living donor as described by Visser and Apperloo et al. four months prior to transplantation[34,35]. During the measurements, donors were seated in a quiet room in a semi supine position. After drawing a blank blood sample, the priming solution containing 0.04 mL/kg body weight of the infusion solution (0.04 MBq of ^{125}I-iothalamate and 0.03 MBq of ^{131}I-hippurate per mL saline) plus an extra of 0.06 MBq of ^{125}I-iothalamate was given, followed by constant infusion at 12 mL/h. To attain stable plasma concentrations of both tracers, a 2-hour stabilization period followed, after which the clearance periods start. Clearances were measured over the next 2 hours and calculated as (U*V)/P and (I*V)/P, respectively. U*V represents the urinary excretion of the tracer, I*V represents the infusion rate of the tracer and P represents the tracer value in plasma at the end of each clearance period. GFR was calculated from U*V/P or ^{125}I-iothalamate and corrected for voiding errors by multiplying the urinary clearance of ^{125}I-iothalamate with the ratio of the plasma and urinary clearance of ^{131}I-hippurate. The day-to-day variability for GFR is 2.5%. Body surface area (BSA) was calculated as according to Dubois[36]. GFR was normalized by dividing the raw sample by BSA and multiplying it with 1.73, giving $GFR/_{BSA}$.

Clinical data

We measured body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) and serum creatinine in the living donor prior to transplantation during the GFR measurements. For DBD donors these parameters were recorded from the Eurotransplant donation forms.

Statistical analysis

Analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL). For comparison of two groups, Mann Whitney U test was performed in case of continuous variables, the Chi-square test for dichotomous variables and the Fisher's Exact test for the history of diabetes mellitus. Results are presented as mean ± SD (standard deviation). All eight histopathological injury parameters showed a skewed distribution. Biopsies of different donor types were not matched to analyze the difference in histopathological injury. To evaluate associations of these injury parameters with donor characteristics, Spearman's correlation coefficients

were calculated on the pooled donor data to determine which variables were significantly associated. Two-sided P-values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Donor characteristics

The characteristics of DBD and LD are shown in table 1. The groups were similar with respect to age at donation, SBP and a history of hypertension. Females were overrepresented in the DBD group compared to the LD (65.4% vs. 45.8%). Compared to DBD donors, LD had significantly higher levels of serum creatinine (78±15 µmol/L vs. 73±25 µmol/L, p=0.002). BMI and DBP at donation were significantly higher in the LD compared to DBD donors (BMI 26±4 kg/m^2 vs. 25±4 kg/m^2, p=0.003; DBP 77±9 mmHg vs. 70±13 mmHg, p<0.001).

Table 1. Characteristics of 131 living donors and 73 DBD donors at the day of their last pre-transplant visit to the out-patient clinic or last pre-transplant data reported by Eurotransplant

Donor type	DBD donors (n = 73)	Living donors (n = 131)	p
Demographics			
Age at donation (years)	53 [44-57]	53 [45-59]	0.51
Female donor, n (%)	65	46	0.006
BMI at donation (kg/m^2)	25 ± 4	26 ± 4	0.003
Underlying illness			
History of hypertension (%)	26	20	0.31
History of diabetes mellitus (%)	6	0	0.007
Hemodynamic parameters			
SBP (mmHg)	130 ± 27	129 ± 15	0.18
DBP (mmHg)	70 ± 13	77 ± 9	<0.001
Renal Function parameters			
Serum creatinine (µmol/L)	73 ± 25	78 ± 15	0.002
GFR (ml/min/1.73 m^2)	NA	117 ± 21	
Death			
Death: CVA	74 %	NA	
Death: trauma/other	26 %	NA	
Duration of brain death (min)	613 [478-740]	NA	

Data are presented as mean ± SD, % or median [interquartile range]. Abbreviations: DBD, deceased brain dead; BMI, body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; GFR, glomerular filtration rate; CVA: cardiovascular accident. P for difference was tested by the Mann-Whitney U test.

Correlations with donor characteristics

Donor age was correlated with IF and FGS (ρ=0.5, p=0.02 and ρ=0.6, p=0.03 resp.) in all biopsies. No correlations of damage parameters with GFR prior to donation (living donors) were found. None of the histological damage parameters correlate with the use of antihypertensive drugs except for the use of calcium antagonists and IF (ß=0.48, p=0.03) in LD.

Correlations of LVD with other histological damage parameters

These correlations in the whole donor group are shown in table 2. LVD mean and LVD at T1 and T2 was associated with macrophage intensity (ρ=0.22, p=0.03; ρ=0.24, p=0.02; ρ=0.27, p=0.001 resp.). LVD measured at T1 was associated with arterial intima thickness (ρ=0.22, p=0.04). LVD T3 was associated with FGS (ρ=0.21, p=0.01). Mean LVD was found to be associated with mean vascular hyalinosis (ρ=0.19, p=0.009). At T2, LVD was associated with neutrophilic granulocytes (ρ=0.20, p=0.01). Within the DBD biopsies, LVD was correlated with signs of arteriopathy such as hyalinosis (ρ=0.25, p=0.04), and with markers of interstitial injury such as IF (ρ=0.3, p=0.02).

Difference in pre-existent damage between DBD and LD

Data on al histopathological damage parameters are shown in table 3. The number of interstitial macrophages and α-SMA intensity was higher in DBD donors compared to LD (1.55±1.44 vs. 2.24±2.09, p=0.001; 0.14±0.1 vs. 0.09±0.05, p=0.02; resp.; figure 1). The number of neutrophilic granulocytes and vascular hyalinosis scores were higher as well in DBD donors (0.33±0.35 vs. 0.21±0.27, p=0.007; 0.44±0.67 vs. 0.18±0.42, p=0.003; resp.; figure 1). No significant differences in glomerulosclerosis, IF and arterial intima thickness between both donor types were found (figure 1).

Table 2. Correlations of LVD and histopathological damage parameters based on renal biopsies of kidney transplant donors

	LVD T1 n = 99		LVD T2 n = 159		LVD T3 n = 152		LVD mean	
Parameter	ρ	p	ρ	p	ρ	p	ρ	p
Macrophage intensity (number/10000 μm²)	0.24	**0.02**	0.27	**0.001**	0.03	0.78	0.22	**0.03**
Mean arterial intima thickness (% of media thickness)	0.22	**0.04**	0.041	0.62	0.08	0.32	0.14	0.07
Glomerulosclerosis (% of total glomeruli)	0.002	0.99	0.13	0.10	0.21	**0.01**	0.10	0.17
Interstitial α-SMA (intensity)	0.03	0.76	0.12	0.15	0.09	0.30	0.10	0.17
Mean vascular hyalinosis (number)	0.11	0.27	0.13	0.10	0.09	0.28	0.19	**0.009**
Mean interstitial fibrosis (number)	0.07	0.49	0.07	0.40	0.16	0.06	0.12	0.09
Interstitial neutrophilic granulocytes(number*10000) T2			0.20	**0.01**				

Data are expressed as Spearman's rho (ρ) and p-value.

Table 3. Histological damage parameters at time of donation

Parameter	DBD donors n=73	Living donors n=131	p
Lymphatic vessel density (lymphatic vessel/mm^2)	7.7 ± 5.92	4.61 ± 3.14	0.001
Interstitial α-SMA (intensity)	2.24 ± 2.09	1.55 ± 1.44	0.02
Interstitial macrophages (number)	0.14 ± 0.1	0.09 ± 0.05	0.001
Interstitial neutrophilic granulocytes (number*10000)	0.33 ± 0.35	0.21 ± 0.27	0.007
Glomerulosclerosis (% of total glomeruli)	0.38 ± 0.66	0.50 ± 2.92	0.72
Interstitial fibrosis	0.76 ± 0.60	0.83 ± 0.49	0.59
Vascular hyalinosis	0.44 ± 0.67	0.18 ± 0.42	0.003
Arterial intima thickness (% of media thickness)	42.7 ± 29.4	36.5 ± 15.2	0.27

Data are expressed as mean ± SD. IF, FGS and vascular hyalinosis are expressed in absolute numbers. P for difference was tested by the Mann-Whitney U test.

Table 4. Histological damage parameters at time of donation in of DBD donors separated by cause of death

Parameter	DBD donors CVA n=57	DBD donors Trauma n=16	p
LVD (lymphatic vessel/mm^2)	8.11 ± 6.34	5.78 ± 2.80	0.71
Interstitial α-SMA (intensity)	2.34 ± 2.13	1.95 ± 2.00	0.47
Interstitial macrophages (number)	0.15 ± 0.09	0.12 ± 0.12	0.06
Interstitial neutrophilic granulocytes (number*10000)	0.37 ± 0.38	0.20 ± 0.15	0.27
Glomerulosclerosis (% of total glomeruli)	0.43 ± 0.71	0.19 ± 0.44	0.17
Interstitial fibrosis	0.92 ± 0.56	0.39 ± 0.53	0.001
Vascular hyalinosis	0.50 ± 0.70	0.30 ± 0.57	0.25
Arterial intima thickness (% of media thickness)	48.56 ± 33.29	28.75 ± 4.72	0.001

Data are expressed as mean ± SD. IF, FGS and vascular hyalinosis are expressed in absolute numbers. P for difference was tested by the Mann-Whitney U test.

LVD was markedly higher in DBD donors compared to LD (7.70±5.92 vs. 4.61±3.14, p<0.001, figure 2), and were found around arteries and veins. Almost no lymph vessels were observed in interstitial space far from arterial adventitia.

Secondary analyses in DBD donors separated by cause of death

In table 4, damage scores of the DBD donors are shown per cause of death. LVD was not significantly different in DBD donors that died from a CVA compared to trauma or other causes. IF and arterial intima thickness were higher in the group that died as a result of a CVA (0.92±0.56 vs. 0.39±0.53; 48.56±33.29 vs. 28.75±4.72, resp.; both p<0.05).

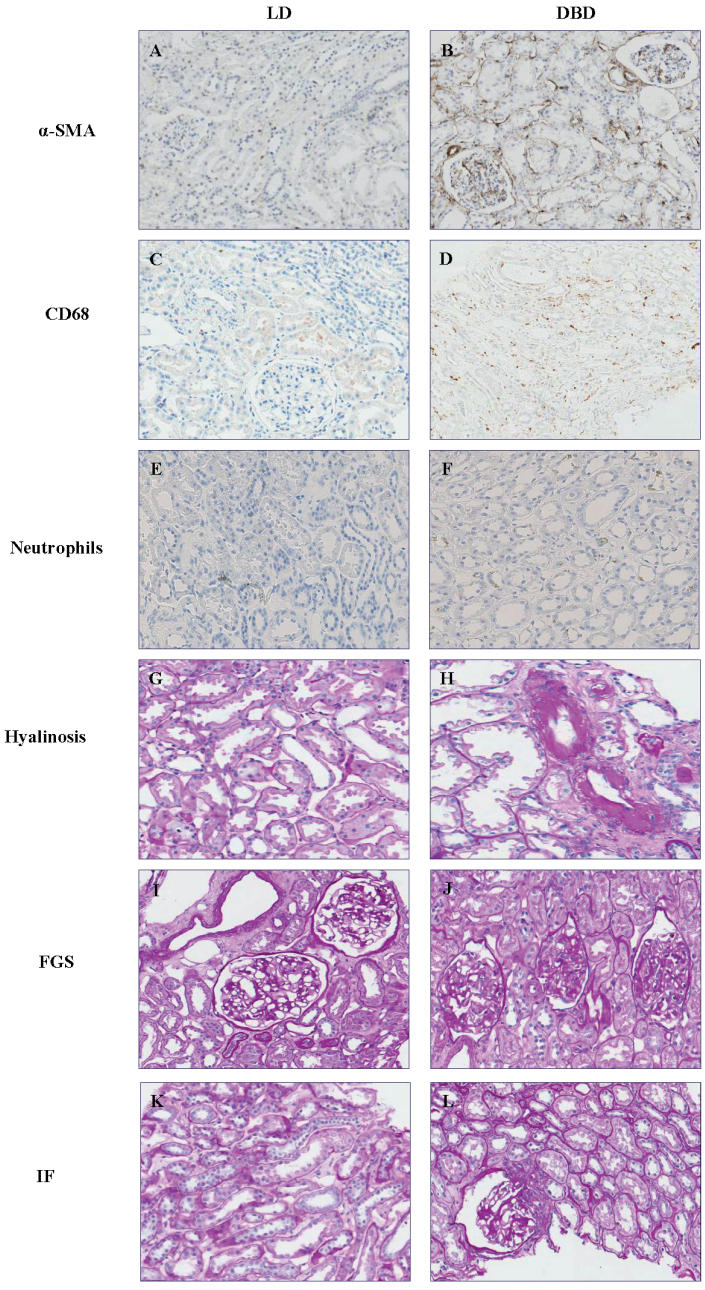

Figure 1. Representative image of stained cortical kidney sections of living and DBD donors. The images demonstrate an increase in the α-SMA expression (A, B), number of interstitial macrophages (C, D), numbers of neutrophilic granulocytes (E, F) and vascular hyalinosis (G, H) in DBD donor biopsies. The extent of FGS (I, J) and interstitial fibrosis (K, L) showed an increase in some samples of DBD, however, when comparing the whole series of biopsies between LD and DBD, there was no significant difference.

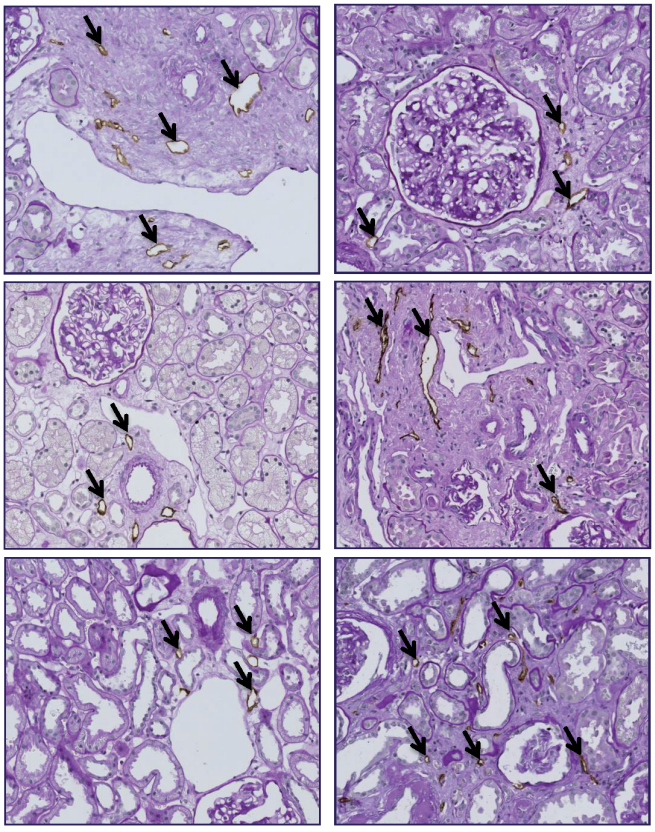

Figure 2. Representative image of double staining for PAS and D2-40 of cortical kidney sections of DBD donors. The arrows indicate the lymphatic vessels in the cortical region of kidney biopsy sections.

DISCUSSION

To our knowledge, this is the first study showing that the number of lymphatic vessels is significantly higher in pretransplant kidney biopsies of DBD compared to LD. In line with the number of LVs, pro-fibrotic, vascular and inflammatory damage in the pretransplant DBD kidney biopsies were also higher. The prominent pre-existent renal damage in kidneys from DBD donors compared with kidneys from LD is supported by several other studies. However, this evaluation has not been performed in such a large cohort before, thereby providing a more complete understanding of the differences in donor kidney quality[6,7].

The role of lymphangiogenesis in organ transplantation is still under debate. Upon renal transplantation, lymphangiogenesis has been observed; however, little is known about the role of lymphangiogenesis at time of donation. LVD was not different in

biopsies collected from transplanted kidneys with interstitial fibrosis and tubular atrophy, independently of rejection[25]. However, more LVs around cellular infiltrates were correlated with superior graft function at one year after renal transplantation[24]. In our study, interstitial (mostly periarterial region) LVD in (pre)transplant biopsies was associated with arteriopathy and tubulointerstitial injury and not with fibrotic parameters. The living donors were somehow selected, since they had higher diastolic pressure, lower kidney function and higher BMI compared to the DBD donors. In their kidneys, this is histologically reflected by the high values of glomerulosclerosis, IF and arterial intima thickness. Despite this, all inflammatory parameters and LVD were low in these kidneys. This already suggests that pre-existent fibrotic responses are rather independent of inflammation and lymphangiogenesis.

As CVA is the consequence of existing vasculopathy, of which vascular hyalinosis is an important sign, we expected to find a difference in hyalinosis between the two DBD donor types but our analysis did not demonstrate that. This is possibly due to the low number of trauma-DBDs. On the other hand, the correlation we found between vascular hyalinosis and mean LVD seems reasonable. Hyalinosis is a sign of vascular leakage and fluid drainage, and on the other hand, accumulation of interstitial fluid and tissue swelling is one of the most potent lymphangiogenic stimuli in order to increase the key functional task of LVs in draining that interstitial fluid[26]. Our data suggest that lymphangiogenesis, as part of a complex tubulointerstitial tissue remodeling program, is already present in donor biopsies prior to implantation. Whether interventional therapy aiming at the modulation of lymphatic numbers and/or functions can improve functional outcome after transplantation remains to be determined.

In line with several other studies we found that donor age is correlated with IF and FGS[15,17,23]. Interestingly, we found a difference in vascular hyalinosis between the two types of DBD donors. This can not completely be the result of the difference in brain death origin. Perhaps other clinical factors which we did not collect, such as the number of antihypertensives used, play a role. No significant differences were found between LVD in DBD donors that died as a result of a CVA compared to DBD donors that died due to a trauma. However, the increased intensity of IF and arterial intima thickness in DBD donors that died as a result of a CVA compared to DBD donors that died of trauma, stresses the importance of these morphological damage that originated before brain death. This is illustrated by the observation that no significant difference in these parameters between DBD and LD are present, while LVD is significantly higher in DBD.

Several limitations of our study deserve acknowledgement. First, it is based on one single European center with possible region-specific demographic characteristics. Our center does not include an absolute cutoff for donor age, however, only 22.5% of the donors were aged above 60 years. Only 11% of the donors had a BMI exceeding 30 kg/m2. Since in our center kidneys from LD with a history of diabetes

are not used, one might question whether the significantly higher score of vascular hyalinosis in DBD donors is a reflection of diabetic vasculopathy. However, we did not find higher scores of glomerulosclerosis and arterial intima thickness in the DBD donors. Taking our observations together, some of the results we found in our single center transplant population are comparable to those of other centers while some new findings regarding LVD and the histopathology in DBD were presented as well. The different donor types in our study population make it difficult to make overall comparisons with other centers. However, our results highlight the multifactorial origin of histological damage in kidney transplant biopsies.

In conclusion, this analysis, which incorporates and confirms the separate observations of previous studies, includes several new observations. Our study demonstrates that LVD, α-SMA, macrophages, neutrophilic granulocytes and vascular hyalinosis are increased in the DBD donor at time of donation compared to living donors. We showed the relevance of LVD in pre-existent renal damage in kidney transplantation biopsies, which was mainly peri-adventitial localized, mostly seen in DBD-CVA donor kidneys and associated with hyalinosis and renal inflammation, but not with fibrosis. Validation of LVD in the assessment of kidney quality before transplantation needs to be determined prospectively in an independent large cohort of kidney transplant biopsies. Furthermore, regarding the DBD donors, it needs to be evaluated whether a separate scoring approach predicting transplant outcome is warranted.

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CHAPTER 6

Angiopoietin-2 single nucleotide polymorphisms affect graft survival after renal transplantation

WH Westendorp
WG van Rijt
MA Seelen
H Snieder
J Damman
J van den Born
MH de Borst
MCRF van Dijk
BG Hepkema
JL Hillebrands
J Niesing
G Navis
RJ Ploeg
SJL Bakker
H van Goor
HGD Leuvenink

ABSTRACT

Background

Elevated angiopoietin-2 (Ang2) levels in renal transplant recipients have been associated with cardiovascular events and mortality in dialysis patients. Upon renal transplantation, Ang2 levels have been associated with graft failure and mortality. In this study, we investigated the role of the functional Ang2 single nucleotide polymorphisms (SNPs) in deceased donor renal transplantation.

Methods

The genotypes of rs2442635 rs2442468, rs2515435 and rs2916702 were determined in deceased donors and recipients of a deceased donor kidney from a total of 1270 transplantations. Ang2 genotypic variations were associated in recipients or donors with death censored graft survival, primary non-function (PNF), delayed graft function (DGF), acute rejection and all-cause mortality.

Results

The homozygote genotype of rs2442468 in recipients was associated with death censored graft survival compared to the homozygote of the major allele. On the other hand, the heterozygote and homozygote genotypes of Ang2 SNPs rs2442635, rs2515435 and rs2916702 in recipients were associated with death censored graft survival. The homozygote genotype of rs2442468 in the donors was associated with a reduced incidence of PNF. The heterozygote and homozygote genotypes of rs2442635, rs2515435 and rs2916702 were associated with an increased risk of PNF compared to the homozygote of the major allele. Compared to the homozygote of the major allele, the heterozygote genotype of rs2916702 in recipients was significantly associated with increased mortality. The genetic Ang2 profile does not influence DGF.

Conclusion

This is the first study showing that the genetic Ang2 profile of the deceased donor and recipient is associated with transplantation outcome. This finding needs to be confirmed by a replication study.

INTRODUCTION

Both angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2), important regulators of angiogenesis, play an essential role in the regulation of vascular stability and are involved in the inflammatory balance provoked by renal ischemia/reperfusion injury (IRI)[1,2]. They bind to the extracellular domain of the tyrosine kinase Tie2 receptor, which is expressed by endothelial and hemopoietic stem cells[3-5]. Precursor pericytes and vascular smooth-muscle cells (SMCs) are responsible for the constant production and release of Ang1, while Ang2 is stored in Weibel Palade bodies (WPB) in the endothelial cells which is rapidly released upon endothelial activation[6-8]. In the healthy adult vasculature, a constant Ang1-mediated Tie2 phosphorylation seems to control and maintain vascular quiescence, damping inflammation and inhibiting endothelial apoptosis, while Ang2 boosts endothelial activation and dysfunction[9-11].

Endothelial dysfunction is an important mediator of IRI and brain death-induced inflammation[12,13]. In the development of allograft vasculopathy after renal transplantation, a critical role for angiopoietins and their Tie2 receptor is proposed given their known role in maintenance of the vascular integrity[14]. Intervention in this mechanism may ameliorate renal IRI as human renal IRI induces endothelial activation after reperfusion, reflected by Ang2 release from the kidney[1,2]. Thus, imbalance in favor of Ang2 causes endothelial activation and dysfunction[15,16]. However, the precise underlying mechanism inducing release of the endothelium stabilizing Ang1 and the destabilizing Ang2 remains unclear since a one on one antagonistic functioning of these angiopoietins is being criticized as Ang2 seems to be the more dynamic player responding rapidly to the internal environment[17,18]. After renal IRI an increased release of Ang2 by the kidney was demonstrated in grafts of living kidney donors and renal transplant recipients (RTR) while the release of Ang1 was not affected[2,19]. In addition, inhibiting Ang2 is likely to prevent transplant IRI in rats[20]. This indicates that increased levels of circulating Ang2 may play an important role in renal allograft vasculopathy. Therefore, it is important to increase understanding of the role of angiopoietin mediated signaling in renal transplantation.

Circulating Ang2 levels have been associated with poor outcome after trauma, sepsis, pancreatitis, chronic kidney disease (CKD) and transplantation[21-24]. Increased Ang2 levels were associated with increased mortality in a RTR case-cohort study[19]. Furthermore, a correlation of increased Ang2 levels with increased C-Reactive Protein levels (CRP), N-terminal pro-Brain Natriuretic Peptide (Nt-pro-BNP) and proteinuria was demonstrated. Ang2 levels correlated negatively with estimated Glomerular Filtration Rate (eGFR), hemoglobin levels and albumin concentration[14] which underlines the importance of Ang2 in (renal) inflammatory conditions.

Ang2, consisting of 496 amino acids and compromising nine exons and eight introns, encodes the Ang2 protein growth factor[25] and is located on chromosome 8q23 which has been found to be highly polymorphic[26,27]. Single nucleotide polymorphisms (SNPs) have been identified in the Ang2 gene which may affect Ang2 gene expression or vascular angiogenesis[28]. So far, Ang2 gene polymorphism was studied in idiopathic recurrent miscarriage, unexplained intrauterine fetal death and gynecologic cancers[29-31]. In the literature, Ang2 SNPs are associated with the development of acute lung injury and increased risk of acute respiratory distress syndrome[32,33]. However, the functional effect of these Ang2 SNPs has not been demonstrated. The role of Ang2 SNPs in the development of renal disease or the effect on outcome after renal transplantation has never been investigated. In this study, we therefore study the effect of Ang2 SNPs on outcome after deceased renal transplantation since circulating Ang2 has been found to associate with graft failure and mortality in deceased donor-RTR. Secondarily, we tested the hypothesis that Ang2 SNPs are involved in the development of end-stage renal disease (ESRD).

MATERIALS & METHODS

Study population

Transplantations (n=1430) between 1993 and 2008 were retrospectively selected for our genetic study[34]. Exclusion criteria were absence of DNA, simultaneous kidney/pancreas or kidney/liver transplantation, loss of follow-up, technical problems or living donor renal transplantation. The inclusion and number of recipients and donors is shown in supplementary table 1, final analysis were performed with the deceased donor inclusions. Differences in numbers of patients are explained by technical problems in the SNP analysis. After transplantation time to graft failure was monitored and censored for death with a functioning graft. Graft failure was defined as return to dialysis or re-transplantation. Clinical parameters of donors and recipients were retrieved from medical files and documented. The study protocol was approved by the institutional review board of the University Medical Center Groningen. All recipients signed written informed consent. According to the Dutch Transplantation Law this was not required for deceased donors. The Institutional Review Board approved the study protocol, which was in adherence to the Declaration of Helsinki.

SNP selection

The following Ang2 tagging SNPs were selected: rs2442468, rs2442635, rs2515435 and rs2916702. This was based on recent publications selecting them on their genomic region, pairwise tagging of the HapMap population[27], minor allele frequency and the measured and calculated pairwise linkage disequilibrium[32,33]. The outcome of the mutated homozygote and heterozygote genotypes were compared to the homozygote of the major alle genotype (reference group).

Study endpoints

The primary end point of outcome after transplantation was death censored graft survival, defined as the need for dialysis or re-transplantation. Furthermore, the effects on primary non-function (PNF), delayed graft function (DGF), acute rejection and all-cause mortality were analyzed. PNF was defined as no graft function after transplantation and DGF was defined as need for dialysis within the first week after transplantation. For analysis of death censored graft survival, DGF and acute rejection, PNF kidney grafts were excluded as these kidneys never functioned.

DNA isolation and SNP analysis

DNA isolation and subsequent SNP analysis of the REGaTTA cohort has been described earlier[34-37]. Peripheral whole blood of recipients or lymphatic tissue of deceased donors was used for DNA extraction by a commercial kit following manufacturer's instructions. DNA concentration was calculated by the NanoDrop nucleic acid application. Isolation procedures were repeated if the concentration of DNA was too low. For SNP genotyping, the Illumina VeraCode GoldenGate assay kit (Illumina, San Diego, CA, USA) was used according to the terms of use. Genotype clustering and calling were performed using BeadStudio Software (Illumina).

Statistical analysis

Data were analyzed with SPSS 20.0 (SPSS Inc., Chicago, USA). Data were presented as mean ± standard deviation (SD) or median [interquartile range] depending on the distribution. The Hardy-Weinberg equilibrium was tested in donors and recipients. Patient characteristics were compared by Mann-Whitney U (continuous data) -or Chi-square test (binary data). The effect of Ang2 SNPs on death censored graft survival and all-cause mortality was initially analyzed and plotted by Kaplan-Meier analyses. The TT genotype was used as reference group meaning that the outcome of the GT- and GG genotype was compared to the TT genotype. Estimated survival was defined as the area under the survival curve. Cox regression analyses were performed to adjust for a priori defined factors, potentially influencing graft survival. These factors include donor age, donor gender, donor type, recipient age, recipient gender, cold ischemia time and the number of transplantations. The effect of the Ang2 SNPs on PNF, DGF and acute rejection was analyzed by binary logistic regression, adjusting for the potentially influencing factors mentioned above.

RESULTS

The characteristics of deceased donor renal transplantations are shown in table 1. In both donors and recipients, the SNP distribution was tested according to the Hardy Weinberg equilibrium. The distribution of rs2442635 in donor kidneys and the distribution of rs2916702 in recipients was skewed (supplementary data – table 2).

Table 1. Transplantation characteristics

Parameter	Deceased donor kidney Tx (989)	Living donor kidney Tx (282)
Gender recipients: male, n (%)	573 (58)	166 (59)
Age recipients, years[a]	51 [40-60]	43 [31-55]
Gender donors: male, n (%)	519 (53)	126 (45)
Age donors, years[a]	46 [32-55]	50 [43-57]
Donor type DBD, n(%) DCD, n(%)	 787 (80) 202 (20)	
Cold ischemia time[a]	1200 [960-1440]	152 [120-182]
Tx without HLA mismatch, n (%)	231 (23)	
Previous transplant, n (%)	107 (11)	21 (7)
PNF, n (%)		
DGF, n (%)	400 (40)	15 (5.3)
Acute rejection, n (%)		
Death censored graft failure	181 (18)	24 (9)
All-cause mortality, n (%)	175 (18)	17 (6)

[a]Median [interquartile range]. Abbreviations: DBD: deceased brain death; DCD: deceased cardiac death; Tx: transplantation; PNF: primary non-function; DGF: delayed graft function.

The effect of Ang2 SNPs on development of end-stage renal disease

The distribution of the Ang2 SNPs between recipients and donors has been compared to determine the role of these SNPs in the development of ESRD (figure 1). No difference in the distribution of rs2442468 was observed. However, rs2442635 and rs2515435 tended to be aberrantly distributed. This skewed distribution between donors and recipients was significant for rs2916702.

The effect of Ang2 SNPs on death censored graft survival

The homozygote genotype of rs2442468 in recipients was associated with reduced death censored graft survival compared to the homozygote of the major allele (table 2). On the other hand, the heterozygote and homozygote genotypes of Ang2 SNPs rs2442635, rs2515435 and rs2916702 in recipients were associated with improved death censored graft survival compared to the homozygote of the major allele (table 2). This association was significant for all heterozygote genotypes and the homozygote genotypes of rs2442635 and rs2515435. A tendency was observed between the homozygote genotype of rs2916702 and the reference group. No differences in death censored graft survival were observed between genotypes of the Ang2 SNPs in the donor kidneys (table 2). The effect on death censored graft survival is illustrated in figure 2.

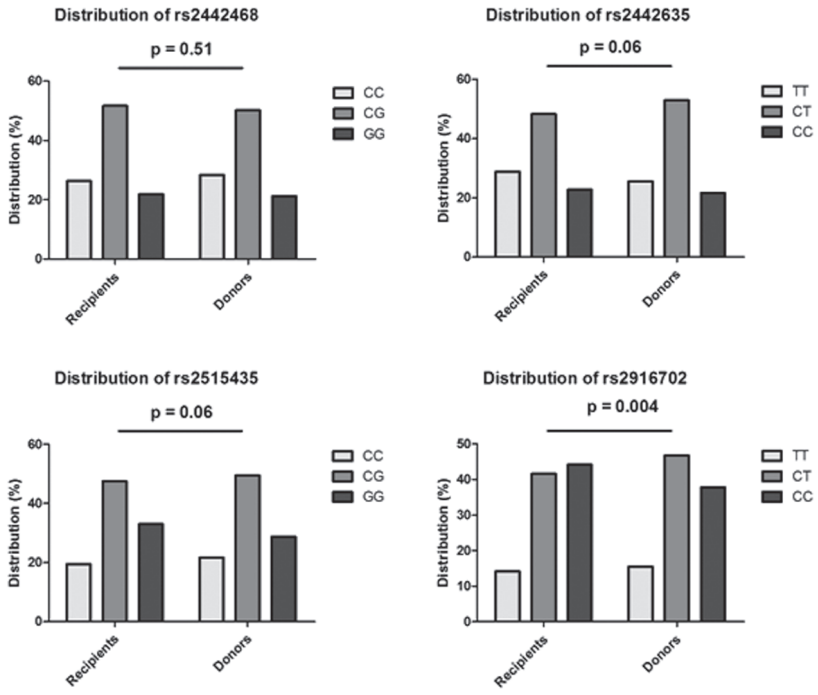

Figure 1. Distribution of four Ang2 SNPs in kidney donors and recipients. No differences in the distribution of the Ang2 SNPs rs2442468, rs2442635 and rs2515435 were observed in kidney donors and recipients. The distribution of the Ang2 SNP rs2916702 did differ between kidney donors and recipients (p=0.004).

Table 2. The effect of donor kidney and recipient genotypes of angiopoietin-2 SNPs on death censored graft survival after deceased donor renal transplantation

Recipient SNP	Homozygote (Reference)	Heterozygote	p	Homozygote (SNP)	p
rs2442468 (n)	CC (249)	CG (482)		GG (200)	
Est. survival (years, 95% CI) [a]	14.2 (13.5-14.9)	14.1 (13.6-14.7)	0.95	**13.0 (12.0-13.9)**	0.10
Hazard ratio (95% CI) [b]	1.00	0.99 (0.65-1.51)		1.47 (0.93-2.30)	
rs2442635 (n)	TT (268)	**CT (446)**		**CC (215)**	
Est. survival (years, 95% CI) [a]	12.8 (12.0-13.6)	**14.3 (13.8-14.9)**	**0.00**	**14.4 (13.6-15.1)**	**0.03**
Hazard ratio (95% CI) [b]	1.00	**0.54 (0.37-0.78)**		**0.60 (0.38-0.94)**	
rs2515435 (n)	CC (307)	**CG (439)**		**GG (183)**	
Est. survival (years, 95% CI) [a]	13.1 (12.3-13.8)	**14.2 (13.7-14.8)**	**0.01**	**14.5 (13.7-15.3)**	**0.03**
Hazard ratio (95% CI) [b]	1.00	**0.62 (0.43-0.89)**		**0.59 (0.36-0.95)**	
rs2916702 (n)	TT (413)	**CT (376)**		CC (137)	
Est. survival (years, 95% CI) [a]	13.3 (12.6-13.9)	**14.5 (13.9-15.0)**	**0.02**	14.1 (13.2-15.0)	0.17
Hazard ratio (95% CI) [b]	1.00	**0.69 (0.43-0.92)**		0.69 (0.41-1.16)	

Table 2. The effect of donor kidney and recipient genotypes of angiopoietin-2 SNPs on death censored graft survival after deceased donor renal transplantation (*Continued*)

Donor kidney SNP	Homozygote (Reference)	Heterozygote	p	Homozygote (SNP)	p
rs2442468 (n)	CC (261)	CG (464)		GG (204)	
Est. survival (years, 95% CI) [a]	13.7 (12.9-14.4)	13.9 (13.3-14.5)	0.91	14.2 (13.3-15.0)	0.65
Hazard ratio (95% CI) [b]	1.00	0.98 (0.66-1.44)		0.90 (0.56-1.44)	
rs2442635 (n)	TT (244)	CT (487)		CC (201)	
Est. survival (years, 95% CI) [a]	14.1 (13.3-14.9)	13.9 (13.4-14.5)	0.92	13.5 (12.6-14.4)	0.54
Hazard ratio (95% CI) [b]	1.00	1.02 (0.68-1.55)		1.16 (0.72-1.87)	
rs2515435 (n)	CC (272)	CG (452)		GG (204)	
Est. survival (years, 95% CI) [a]	14.1 (13.3-14.8)	13.9 (13.4-14.5)	0.95	13.5 (12.6-14.4)	0.56
Hazard ratio (95% CI) [b]	1.00	0.99 (0.66-1.47)		1.15 (0.72-1.82)	
rs2916702 (n)	TT (360)	CT (426)		CC (143)	
Est. survival (years, 95% CI) [a]	14.1 (13.5-14.8)	13.7 (13.1-14.3)	0.83	13.9 (12.8-14.9)	0.99
Hazard ratio (95% CI) [b]	1.00	1.04 (0.72-1.51)		1.00 (0.61-1.67)	

Abbreviations: Est.: estimated; CI: confidence interval.
[a]Kaplan-meier survival analysis with log-rank test. Primary non-function grafts were excluded.
[b]Cox regression analysis adjusted for donor age, donor gender, donor type, recipient age, recipient gender, HLA mismatch, cold ischemia time, number of transplantation. Primary non-function grafts were excluded.

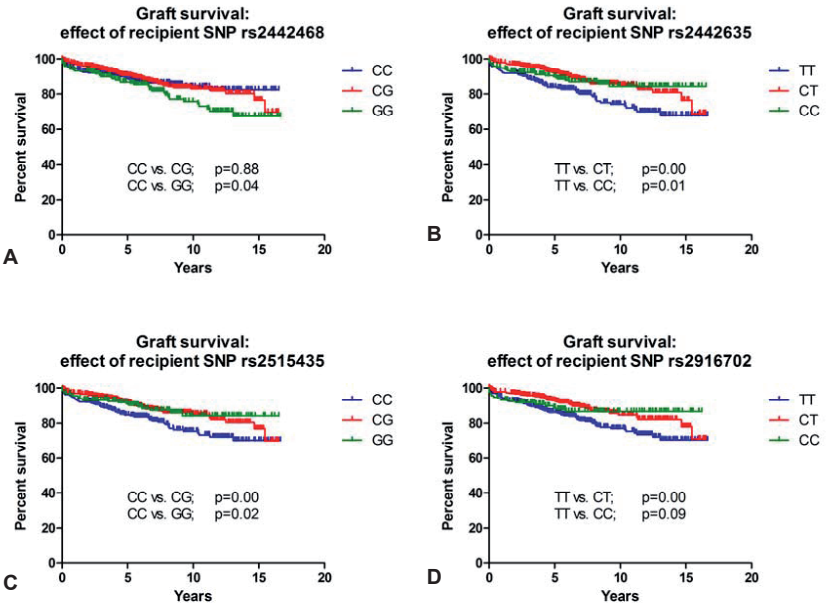

Figure 2. Effect of Ang2 SNPs in renal transplant recipients on death censored graft survival. A) Estimated mean graft survival of CC recipient genotype is superior to the GG genotype (p=0.04). B) Estimated mean graft survival of CC recipient genotype is superior to the TT genotype (p=0.01) and the estimated mean graft survival of CT recipient genotype is superior to the TT genotype (p<0.01). C) Estimated mean graft survival of GG recipient genotype is superior to the CC genotype (p=0.02) and the estimated mean graft survival of CG recipient genotype is superior to the CC genotype (p<0.01). D) Estimated mean graft survival of CT recipient genotype is superior to the TT genotype (p<0.01).

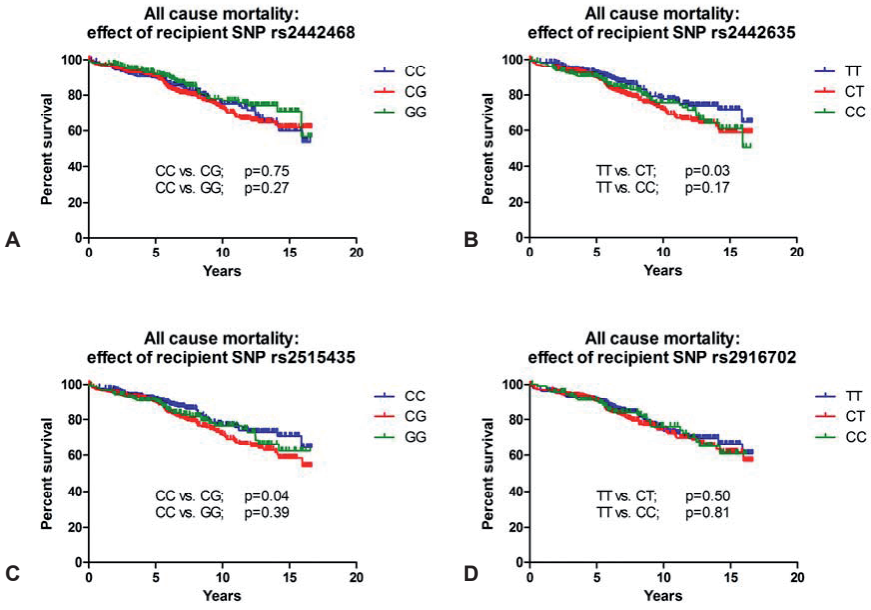

Figure 3. Effect of Ang2 SNPs on all-cause mortality in renal transplant recipients. A) No differences in patient survival were observed between recipient genotypes. B) Estimated mean patient survival of TT recipient genotype is superior to the CT genotype (p=0.03). C) Estimated mean patient survival of CC recipient genotype is superior to the CG genotype (p=0.04). D) No differences in patient survival were observed between recipient genotypes.

The effect of Ang2 SNPs on primary non-function and delayed graft function

In recipients, the Ang2 SNPs were not associated with PNF, while in donor kidneys significant associations were observed. The homozygote genotype of rs2442468 in the donors was associated with a reduced incidence of PNF. However, the heterozygote and homozygote genotypes of rs2442635, rs2515435 and rs2916702 were associated with an increased risk of PNF compared to the homozygote of the major allele. For delayed graft function no relevant differences between the genotypes of the Ang2 SNPs in recipients or donor kidneys were observed, as only the heterozygote genotype of rs2442468 in recipients was significantly associated with reduced DGF (table 4).

The effect of Ang2 SNPs on all-cause mortality

No relevant differences in association with mortality after transplantation between the genotypes of the Ang2 SNPs in recipients or donor kidneys were observed, as only the heterozygote genotype of rs2916702 in recipients was significantly associated with increased mortality (table 5).

Table 3. The effect of donor kidney and recipient genotypes of angiopoietin-2 SNPs on primary non-function after deceased donor renal transplantation

Recipient SNP	Homozygote (Reference)	Heterozygote	p	Homozygote (SNP)	p
rs2442468 (n)	CC (264)	CG (514)		GG (210)	
Incidence of PNF	5.7 %	6.2 %	0.74	4.8 %	0.45
Odds ratio (95% CI) [a]	1.00	1.12 (0.59-2.13)		0.71 (0.29-1.73)	
rs2442635 (n)	TT (286)	CT (471)		CC (229)	
Incidence of PNF	6.3 %	5.3 %	0.85	6.1 %	0.96
Odds ratio (95% CI) [a]	1.00	0.94 (0.49-1.82)		1.02 (0.48-2.18)	
rs2515435 (n)	CC (329)	CG (463)		GG (194)	
Incidence of PNF	6.7 %	5.2 %	0.54	5.7 %	0.69
Odds ratio (95% CI) [a]	1.00	0.82 (0.44-1.54)		0.85 (0.39-1.86)	
rs2916702 (n)	TT (439)	CT (401)		CC (143)	
Incidence of PNF	5.9 %	6.2 %	0.61	4.2 %	0.52
Odds ratio (95% CI) [a]	1.00	1.17 (0.65-2.11)		0.74 (0.29-1.87)	
Donor kidney SNP	**Homozygote (Reference)**	**Heterozygote**	**p**	**Homozygote (SNP)**	**p**
rs2442468 (n)	CC (283)	CG (496)		**GG (209)**	
Incidence of PNF	7.8 %	6.0 %	0.53	**2.4 %**	**0.02**
Odds ratio (95% CI) [a]	1.00	0.83 (0.46-1.50)		**0.27 (0.09-0.79)**	
rs2442635 (n)	TT (249)	**CT (520)**		**CC (220)**	
Incidence of PNF	1.5 %	**5.4 %**	**0.01**	**6.9 %**	**0.01**
Odds ratio (95% CI) [a]	1.00	**3.9 (1.36-11.3)**		**4.83 (1.59-14.6)**	
rs2515435 (n)	CC (278)	**CG (484)**		**GG (223)**	
Incidence of PNF	2.2 %	**6.6 %**	**0.01**	**8.5 %**	**0.01**
Odds ratio (95% CI) [a]	1.00	**3.56 (1.36-9.32)**		**4.10 (1.48-11.3)**	
rs2916702 (n)	TT (370)	**CT (458)**		**CC (158)**	
Incidence of PNF	2.7 %	**7.0 %**	**0.00**	**9.5 %**	**0.00**
Odds ratio (95% CI) [a]	1.00	**3.22 (1.46-7.15)**		**4.06 (1.66-9.95)**	

Abbreviations: PNF: primary non-function; CI: confidence interval.
[a]Binary logistic regression adjusted for donor age, donor gender, donor type, recipient age, recipient gender, HLA mismatch, cold ischemia time, number of transplantation.

Table 4. The effect of donor kidney and recipient genotypes of angiopoietin-2 SNPs on delayed graft function after deceased donor renal transplantation

Recipient SNP	Homozygote (Reference)	Heterozygote	p	Homozygote (SNP)	p
rs2442468 (n)	CC (249)	**CG (428)**		GG (200)	
Incidence of DGF	42.6 %	**33.0 %**	**0.03**	41.5 %	0.94
Odds ratio (95% CI) [a]	1.00	**0.66 (0.46-0.96)**		0.96 (0.61-1.49)	
rs2442635 (n)	TT (268)	CT (446)		CC (215)	
Incidence of DGF	40.7 %	32.5 %	0.08	42.8 %	0.72
Odds ratio (95% CI) [a]	1.00	0.72 (0.50-1.04)		1.08 (0.71-1.66)	
rs2515435 (n)	CC (307)	CG (439)		GG (183)	
Incidence of DGF	39.1 %	34.2 %	0.38	42.1 %	0.52
Odds ratio (95% CI) [a]	1.00	0.85 (0.60-1.21)		1.15 (0.75-1.79)	
rs2916702 (n)	TT (413)	CT (376)		CC (137)	
Incidence of DGF	38.5 %	35.6 %	0.64	39.4 %	0.87
Odds ratio (95% CI) [a]	1.00	0.92 (0.66-1.29)		1.04 (0.65-1.66)	
Donor kidney SNP	**Homozygote (Reference)**	**Heterozygote**	**p**	**Homozygote (SNP)**	**p**
rs2442468 (n)	CC (261)	CG (466)		GG (204)	
Incidence of DGF	34.1 %	38.4 %	0.12	39.2 %	0.45
Odds ratio (95% CI) [a]	1.00	1.35 (0.93-1.95)		1.19 (0.76-1.85)	
rs2442635 (n)	TT (244)	CT (487)		CC (201)	
Incidence of DGF	38.9 %	37.2 %	0.66	35.8 %	0.43
Odds ratio (95% CI) [a]	1.00	1.09 (0.75-1.58)		0.83 (0.53-1.31)	
rs2515435 (n)	CC (272)	CG (452)		GG (204)	
Incidence of DGF	39.0 %	38.7 %	0.79	32.8 %	0.08
Odds ratio (95% CI) [a]	1.00	1.05 (0.73-1.50)		0.67 (0.43-1.04)	
rs2916702 (n)	TT (360)	CT (426)		CC (143)	
Incidence of DGF	37.8 %	38.5 %	0.99	31.5 %	0.18
Odds ratio (95% CI) [a]	1.00	1.10 (0.79-1.55)		0.72 (0.45-1.16)	

Abbreviations: DGF: delayed graft function; CI: confidence interval.
[a]Binary logistic regression adjusted for donor age, donor gender, donor type, recipient age, recipient gender, HLA mismatch, cold ischemia time, number of transplantation. Primary non-function grafts were excluded.

Table 5. The effect of donor kidney and recipient genotypes of angiopoietin-2 polymorphisms on mortality after deceased donor renal transplantation

Recipient SNP	Homozygote (Reference)	Heterozygote	p	Homozygote (SNP)	p
rs2442468 (n)	CC (264)	CG (514)		GG (210)	
Est. survival (years, 95% CI) [a]	12.2 (11.4-13.0)	12.2 (11.6-12.7)	0.80	12.2 (11.4-13.1)	0.61
Hazard ratio (95% CI) [b]	1.00	1.04 (0.78-1.37)		1.09 (0.78-1.54)	
rs2442635 (n)	TT (286)	CT (471)		CC (229)	
Est. survival (years, 95% CI) [a]	12.2 (11.5-13.0)	12.2 (11.6-12.8)	0.51	12.2 (11.4-13.0)	0.40
Hazard ratio (95% CI) [b]	1.00	0.91 (0.69-1.20)		0.87 (0.63-1.20)	
rs2515435 (n)	CC (329)	CG (463)		GG (194)	
Est. survival (years, 95% CI) [a]	12.3 (11.6-13.0)	12.1 (11.5-12.7)	0.50	12.2 (11.3-13.2)	0.33
Hazard ratio (95% CI) [b]	1.00	0.91 (0.70-1.19)		0.84 (0.60-1.18)	
rs2916702 (n)	TT (439)	**CT (401)**		CC (143)	
Est. survival (years, 95% CI) [a]	12.0 (11.4-12.6)	**12.5 (11.8-13.1)**	**0.01**	12.3 (11.3-13.3)	0.24
Hazard ratio (95% CI) [b]	1.00	**0.72 (0.55-0.93)**		0.81 (0.57-1.15)	
Donor kidney SNP	**Homozygote (Reference)**	**Heterozygote**	**p**	**Homozygote (SNP)**	**p**
rs2442468 (n)	CC (238)	CG (496)		GG (209)	
Est. survival (years, 95% CI) [a]	12.5 (11.7-13.2)	11.9 (11.3-12.4)	0.50	12.4 (11.6-13.3)	0.74
Hazard ratio (95% CI) [b]	1.00	(0.83-1.45)		(0.67-1.33)	
rs2442635 (n)	TT (249)	CT (520)		CC (220)	
Est. survival (years, 95% CI) [a]	12.1 (11.2-12.9)	12.0 (11.5-12.6)	0.58	12.6 (11.7-13.4)	0.85
Hazard ratio (95% CI) [b]	1.00	(0.82-1.44)		(0.68-1.38)	
rs2515435 (n)	CC (278)	CG (484)		GG (223)	
Est. survival (years, 95% CI) [a]	12.0 (11.2-12.8)	12.2 (11.6-12.8)	0.69	12.2 (11.3-13.0)	0.89
Hazard ratio (95% CI) [b]	1.00	1.06 (0.80-1.40)		1.02 (0.73-1.43)	
rs2916702 (n)	TT (370)	CT (458)		CC (158)	
Est. survival (years, 95% CI) [a]	12.0 (11.3-12.6)	12.3 (11.7-12.9)	0.98	12.4 (11.4-13.4)	0.79
Hazard ratio (95% CI) [b]	1.00	(0.77-1.30)		(0.67-1.36)	

Abbreviations: Est.: estimated; CI: confidence interval.
[a]Kaplan-meier survival analysis with log-rank test.
[b]Cox regression analysis adjusted for donor age, donor gender, donor type, recipient age, recipient gender, HLA mismatch, cold ischemia time, number of transplantation.

DISCUSSION

Angiopoietin-2 has been shown to play a substantial role in IRI and outcome after renal transplantation. There is growing evidence that Ang2 is involved in allograft vasculopathy in kidney donors and RTR. This is the first study that demonstrates the role of the Ang2 gene in renal transplantation. Next, Ang2 SNPs were involved in the development of end-stage renal disease. After deceased donor transplantation, these SNPs were associated with improved death censored graft survival. However, the same Ang2 SNPs in the donor kidneys were associated with an increased risk of PNF. This implicates that the effect of the Ang2 gene in renal IRI is different from its role in development of ESRD.

The results of this study would be strengthened by confirmation in an independent cohort. Nevertheless, in this cohort the effect of the Ang2 SNPs on development of end-stage renal disease is evident. The distribution of the Ang2 SNPs is skewed towards the homozygote of the major allele. This means that this homozygote of the major allele increases the risk of end-stage renal disease. Subsequently, the homozygote of the major allele of these SNPs in recipients is associated with impaired graft survival. Thus, both before and after transplantation, recipients with these SNPs are predisposed to develop end-stage renal disease.

In contrast to the role of Ang2 SNPs in the long-term outcome, the heterozygote and homozygote genotypes were associated with an increased risk of PNF compared to the homozygote of the major allele. No effect on the incidence of DGF was observed. However, the effect on PNF indicates that the role in short-term outcome after transplantation is contrary to the long-term outcome. Presumably, the function of Ang2 in renal IRI is opposite to the development of end-stage renal disease.

Circulating Ang2 levels have been reported to predict mortality in renal transplant recipients[19]. Unfortunately, only serum Ang2 levels were determined in this particular study. Testing of the association between Ang2 genotype and mortality or graft failure was not performed. In our cohort, the Odd's ratio for mortality of the heterozygote genotype of rs2916702 and for DGF of the heterozygote genotype of rs2442468 in recipients were significantly reduced compared to the homozygote of the major allele. However, no differences in the incidence of DGF between the two homozygote genotypes were observed. These data are inconsequent and this significant difference is likely the result of a bias. We therefore conclude that the Ang2 SNPs do not affect the incidence of delayed graft function or mortality.

We acknowledge some limitations of our study. First, the single center retrospective design which makes generalization to the community setting difficult. Second, we did not examine the functions of Ang2 genotypes and Ang2 levels, thus the functional significance of Ang2 in deceased donors remains to be defined. Furthermore, we emphasize the need for replication of our findings given the large number of false positive generated in genetic association studies[38]. So to fully understand the disparity between our findings and the role of Ang2, the functionality of these Ang2 SNPs has to be confirmed in other transplantation cohorts, including both genotyping and functional Ang2 expression. However, the association of common Ang2 genetic variation with transplant outcome is biological plausible since an *in vitro* study has suggested that Ang2 gene variations alter gene expression[28].

To conclude, this study shows that the Ang2 genotype of RTR is associated with death censored graft survival after deceased donation. These SNPs were involved in the development of end stage renal disease and PNF. Subsequently, the homozygote of the major allele of these SNPs in RTR is associated with impaired graft survival. Hence, both before and after transplantation, recipients with these SNPs are predisposed to develop ESRD. The genetic Ang2 profile does not influence DGF or mortality after renal transplantation.

Supplementary data - table 1. Included number of patients for each SNP

SNP	Recipient SNP	Donor kidney SNP
rs2442468	1270	1270
rs2442635	1268	1271
rs2515435	1268	1264
rs2916702	1265	1266

Supplementary data - table 2. Hardy Weinberg equilibrium

SNP	Recipients	Donor kidneys
rs2442468	0.19	0.73
rs2442635	0.25	0.03
rs2515435	0.27	0.87
rs2916702	0.003	0.57

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CHAPTER 7

Brain death induced renal injury

WH Westendorp
HGD Leuvenink
RJ Ploeg

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PURPOSE OF THE REVIEW

The considerable demand for donor kidneys against a persisting organ donor shortage has forced most centers to nowadays accept suboptimal donor kidneys. Despite the substantial increase in the past decade in kidney transplantation with grafts retrieved from living donors and after donation from deceased brain dead (DBD) and extended criteria donation (ECD) donors, the supply of donor kidneys still does not meet the actual numbers needed. Moreover, older and more marginal kidney donors following the physiologically abnormal state of brain death do function less and have a shorter graft survival. In this review, we present an overview of the current knowledge of renal injury induced by pathophysiological effects of brain death and its relevance for renal transplant outcome. The better insight in the role of brain death induced renal injury has clearly demonstrated its detrimental effect on outcome but, also, offers new opportunities for donor management and evaluation of new biomarkers to assess kidney graft quality in the brain dead donor. The option to intervene and selectively block or enhance a pathway as well as identify specific parameters for graft quality at time of organ retrieval in the deceased brain dead donor will ultimately benefit early function and long-term survival.

INTRODUCTION

Brain death, in the past also referred to as coma dépassée, was first described in 1959 by the French Mollaret and Goulon[1] as a state of irreversible coma without reflexes after massive cerebral injury that required mechanical ventilation. At that time, no consensus had been reached on the implications of this irreversible coma. In 1968, a committee at Harvard Medical School proposed to add irreversible coma to the death criterion, so that in these cases ventilators could be turned off. Its report established the term of brain death for the first time defining it as a 'permanently nonfunctioning brain', providing diagnostic criteria and establishing brain death legally the equivalent to death[2]. This development has formed since then the legal basis for the possibility to obtain organs for transplantation from deceased patients who are brain dead, however, do still have an intact extracerebral circulation.

To date, the declaration of brain death has been accepted by most societies as a point of no return and has made organ donation possible. From the moment of cerebral injury and certainly after herniation of the brain stem a cascade of events takes place that does affect all potential donor organs. This sequence includes an increase of intracranial pressure following cerebral trauma or cerebrovascular hemorrhage and multiple systemic and hormonal changes. One of the significant brain death related events that affects the quality of grafts-to-be prior to organ retrieval is the profound inflammatory and pro-coagulatory response syndrome.

Brain death injury

To date, the majority of organs is still derived from brain dead donors as the main source used in transplantation. Since the need for organs is significantly larger than the availability of ideal donors, criteria for acceptation of donors have been expanded and led to the use of extended criteria donation (ECD). ECD includes brain dead donors who are older than 60 years, or are aged over 50 years in combination with at least two of the following risk factors: a history of hypertension and a terminal serum creatinine more than 1.5 mg/dl or a cerebrovascular cause of death[3]. The period of donor management after brain death is a dynamic and unphysiological course of events that influences a number of pathophysiological processes in the human body. These processes have been demonstrated both in animal models and during clinical investigations in patients.

Brain death often results from cerebral hemorrhage, trauma, or an anoxic event. When the brain becomes ischemic it begins to swell, which leads to increased intracranial pressure, arterial vasodilatation and further increase of edema and intracranial pressure. When this intracranial pressure exceeds the mean arterial pressure, brain perfusion stops, the pituitary gland is damaged, and its hormone secretion rapidly ceases[4,5].

Ischemia of the hypothalamus activates the sympathetic nervous system and releases a massive amount of catecholamines, which typically causes hypertension. After the 'catecholamine storm', hemodynamics reach a hypotensive phase resulting in hypoperfusion of the body in general and of organs in particular[6,7]. This significant ischemic state is marked by elevated serum lactate levels and should be corrected by fluid resuscitation and, if needed, inotropic support. The initial increase in systemic blood pressure is usually followed by hypoperfusion[8,9]. The autonomic or sympathetic catecholamine storm triggers a cascade of derangements that leads to extensive endothelial cell injury, release of cytokines in the circulation and ischemia in all organs of the body. The endothelial injury in the brain will lead to leakiness of the blood–brain barrier. Dysfunction of the blood–brain barrier then allows cytokines produced in the brain to leak into the serum[10].

Hormonal changes during brain death result in a transient and massive increase in circulating catecholamines as well as a decrease in arginine vasopressin (AVP) due to hypothalamic–pituitary dysfunction leading to diabetes insipidus. In addition, Novitzky *et al*.[9] reported a marked decrease in levels of thyroid hormones and cortisol in human brain death donors. They suggested that hormonal changes are the major cause of mitochondrial dysfunction with impaired energy production at the cellular level. This inhibition of mitochondrial function results in diminished organ function from the loss of energy stores from a rapid loss of circulating Fe^{3+} [11].

Pathophysiological effects of brain death on the kidneys

As a result of cerebral injury and brain death the elevated levels of circulating cytokines induce a local inflammatory response in the kidney[10,12]. Microarray studies have demonstrated upregulated expression of genes related to inflammatory response and reparative mechanisms in kidneys after brain death[8,13]. In addition, NF-kB-activation in kidneys and subsequent expression of genes involved in inflammation as a consequence of brain death has been reported as well. Serum levels of interleukin-6, interleukin-8, interleukin-10, and monocyte chemoattractant protein-1 (MCP-1) are significantly increased after brain death. Mitogenactivated protein kinases (MAP kinases) probably play a major role in the transition from the systemic inflammatory signal to the local inflammatory reaction in the kidney[10].

Since the discovery in 1996, the angiopoietin-Tie ligandreceptor system has been studied extensively in critical illness like sepsis[14-17]. The Ang-Tie ligand-receptor system is crucial in regulating vascular integrity and quiescence. Angiopoietin-1 (Ang1) has anti-inflammatory effects while angiopoietin-2 (Ang2) triggers an inflammatory response by activating the endothelium and inducing permeability. Vascular quiescence is maintained by signaling with an Ang1/Ang2 ratio in favor of endogenous Ang1 when Ang2 post transplantation downregulates[18].

In a small cohort of donation after brain death and living kidney donors, we recently found higher Ang1 and Ang2 levels in brain death donors compared to living donors.

During the brain death period the Ang1/Ang2 ratio was decreased. We also noted that elevated Ang2 levels in the donor were associated with an increased risk of post transplantation rejection and that the decrease in Ang1/Ang2 ratio was associated with an increased risk of delayed graft function (DGF) (unpublished).

The effects of adhesion molecules, leukocyte infiltration, gene expression and stress-related heat shock proteins have been extensively studied in human brain dead kidney donors[19-21]. The results showed an increased presence of the early adhesion molecule E-selectin after brain death, but not of the subsequent adhesion molecules ICAM-1 and VCAM-1. A significant difference between brain death and living donor kidneys in the presence of leukocytes in the interstitium was found as well.

Gene expression of two protective heat shock proteins, heme oxygenase-1 (HO-1) and Hsp70, and the growth factor TGF-β, were measured to assess the kidney's response to stress. Both HO-1 and Hsp70 showed an upregulation during brain death, but TGF-β was not significant activated. In the group of living donor kidney recipients, elevated HO-1 expression in the transplanted kidney had a strongly positive effect on low 1-year and 3-year serum creatinine levels. This finding suggests that brain death causes severe renal damage to such an extent that the protective effect of HO-1 is probably insufficient. Further upregulation of protective heat shock proteins may improve outcome of kidneys retrieved from brain dead donors after transplantation[21].

Because of renal vasoconstriction due to excessive secretion of catecholamines and volume depletion, the kidney is exposed to hypoperfusion and ischemic damage during brain death. Renal inflammatory and degenerative lesions appear on histological examination, including glomerulitis, periglomerulitis, vacuolization, atrophy, and necrosis of proximal and distal tubules, as well as proliferation of arterial intima and glomerular endothelium. Upregulation of circulating cytokines and chemokines, increased endothelial cell expression of adhesion molecules and major histocompatibility classes I and II, as well as greater infiltration of T cells, macrophages, and polymorphonuclear leukocytes into renal parenchyma, result in increased renal innate immunogenicity and subsequent host alloresponsiveness[22].

Our group has demonstrated an immediate procoagulatory and pro-inflammatory activation of vascular endothelium after brain death in kidney donor rats, which is proportional with the duration of brain death. E-selectin and P-selectin, Aa/Bb-fibrinogen mRNA were abruptly progressively upregulated already half an hour after brain death and their levels of expression increased progressively over time. Plasma von Willebrand factor (vWF) was significantly higher after 2 and 4 h brain death. Urine heart-fatty-acid-binding-protein (H-FABP) and N-acetyl-

glucosaminidase (NAG) were used as specific markers of proximal and distal tubular damage and found to be significantly increased after half an hour; with a maximum at 4 h. Oxidative stress was detectable, but only very late, after the establishment of tubular injury[23].

To clarify subclinical pathological changes in the grafted kidney, Kotsch *et al.*[24] investigated messenger RNA (mRNA) gene expression profiles in renal zero-hour biopsies from deceased and living donors. A significant induction was observed of the chemokine receptor seven ligands 19/21 [C–C motif] in the deceased donor group. In addition, in parallel with the induction of the activation marker CD69, significant elevated mRNA levels of the subunits PSMB8, PSMB9, and PSMB10 were detected. Although multiple studies have broadened the insight in brain death induced renal injury during the past years, it still remains unknown yet which exact signal transduction pathways are involved inducing the inflammatory response in the kidney. Further analysis of the expression of multiple genes encoding transcription factors and proteins involved in signal transduction, protection and repair is needed for proper identification.

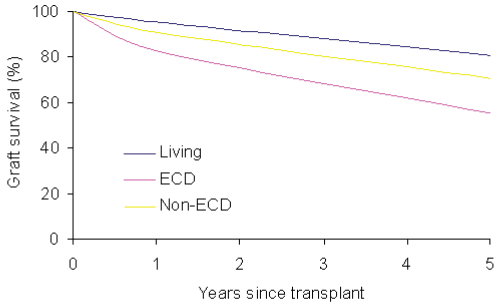
Living donor grafts are associated with lower rates of delayed graft function and better graft survival than kidneys retrieved from brain death donors (figure 1, table 1)[25]. The difference may in part be attributed to the intense release of inflammatory mediators that follows brain death. As a matter of fact, brain death in the donor has been reported as a probable risk factor for developing vascular rejection[26].

Perspectives on donor management and pretreatment

Strategies to reduce the pro-inflammatory status of the graft in the donor are becoming more attractive and may help to improve organ quality and graft function before and after transplantation[27]. The use of pharmacological interventions to counteract the decline of renal function appears to be an elegant and promising approach during brain death.

Due to hypotension and hypoperfusion in the donor during the period of brain death in the ICU, vasopressors such as norepinephrine, dopamine, and vasopressin are normally required[28]. Recently, a randomized controlled trial by Schnuelle *et al.*[29] demonstrated that low-dose dopamine (4mg/kg/min) is beneficial as pretreatment of brain dead kidney donors. Administration of dopamine reduced the need for dialysis from 35.4% to 24.7% within the first week after kidney transplantation.

Furthermore, we have also investigated the impact of brain death on renal function in a rat model of isolated perfused kidneys. Animal groups were pretreated before inducing brain death with erythropoietin (EPO) or an EPO derivate, the carbamylated EPO (cEPO) versus controls. Kidney function was significantly reduced in vehicle-treated rats with brain death but not in the experimental groups and in sham-operated rats. It was shown that cEPO can inhibit the inflammatory response and endothelial activation caused by brain death more effectively than EPO, whereas



Source: UNOS

Figure 1. Graft survival for living-, ECD, and non-ECD-derived renal grafts. Graft survival of kidney transplants performed in the United States over a period of 5 years between 2005 and 2010. Data were obtained from the United Network for Organ Sharing (UNOS).

Table 1. One- and five-year graft survival for living and deceased (ECD/non-ECD) donors following renal transplantation

		One-year survival (%)	Five-year survival (%)
Kidney donor type	Number of transplants	2000-2009	2000-2009
Living	59 627	95.3	80.89
ECD	14 632	82.94	55.39
Non-ECD	75 989	90.99	70.70

Source: UNOS. ECD: extended criteria donation.

both substances do restore kidney function after brain death. Therefore, EPO analogs are interesting candidates for renal protection and clinical intervention[30]. Intervening in the Ang1/Ang2 balance may also be of therapeutical value to improve outcome after brain death renal transplantation. Increasing the Ang1 concentration to restore the balance was found to have a significant anti-inflammatory potential in animal models[31]. Moreover, Ang2 neutralizing reagents have been developed as antiangiogenic tumor drugs and could also be used to decrease the pro-inflammatory status of the donor[32].

Another way to improve organ quality from DBD donors is to alter the activation state of MAP kinases by inhibiting transcription genes involved in transduction pathways[10].

A different and more recent approach to reduce renal injury and enhance repair was advocated by Elkins[33] using inhalational anesthesia in brain dead donors and benefitting the recipient. Peri-operative management and administration of inhalational anesthetics could be beneficial as they will the catecholamine release

that occurs during surgical stimulation at the time of the organ retrieval procedure. Further research is needed to determine how anesthetic preconditioning could be advantageous to preserve the viability of donor kidneys.

To determine the optimal timing of intervention during donor management and induce repair mechanisms, we have studied whether the length of the period of brain death is a risk factor for outcome after kidney transplantation. In a large database analysis, results revealed that longer duration of brain death in the donor after cerebral injury is not detrimental. Longer brain death duration even appears to have a modest beneficial effect on the odds for immediate graft function and 1-year and 3-year grafts survival after kidney transplantation. The duration of brain death had no influence on acute rejection in the first year after transplantation. Thus, instead of 'to rush and retrieve', the attitude 'to relax and repair' with an unhurried and high-quality ICU donor management during brain death prior to organ retrieval is recommended[34].

A different approach to reduce inflammation in the brain dead donor is to induce repair mechanisms using HO-1 induction [31]. HO-1, the enzyme that converts heme to Fe^{2+}, carbon monoxide and biliverdin, has been extensively studied. HO-1 is overexpressed in organs during brain death and the elimination of the excess heme suppresses generation of oxidative radicals and thereby limits the damage associated with those radicals. Therefore, Kotsch *et al.*[35] examined the impact of donor treatment with cobalt protoporphyrin (CoPP) as the selective inducer of HO-1 on organ quality and transplant outcome in a standardized brain death transplant rat model. Recipients of organs from brain dead donors treated with CoPP survived significantly better than those from untreated brain dead donors and intra-graft analysis showed improved histology. Blockade of HO-1 with zinc protoporphyrin (ZnPP) decreased the survival rates comparable with untreated brain dead donors. These results show that HO-1 induction by one single treatment of CoPP in brain dead donors leads to enhanced allograft survival.

Overall, HO-1 and its products of degradation are strong antioxidants that will inhibit cell death, limit apoptosis, and halt aberrant proliferation[36].

Indicators for brain death induced (renal) injury

The fact that nowadays many older and more marginal deceased donor organs are accepted for transplantation determines graft outcome. To assess the chance of immediate graft function in the context of choice of immunosuppressants more specific parameters of the (expected) kidney graft quality at time of organ procurement in the brain death donor are needed. If transplantation success could be better predicted the appropriate matching of organ and recipient characteristics could be significantly improved.

The type 1 transmembrane protein kidney injury molecule-1 (KIM-1 in humans or Kim-1 in rodents) is known to be very specific and upregulated in renal tubular cells

in a variety of injury models[37]. We have analyzed the effect of brain death on the expression of KIM-1 and evaluated its use as a new biomarker in organ donation. In this study KIM-1 distinctly rose as well as gene expressions within 4 h of brain death, while serum creatinine levels remained within the normal range. In addition, patient data showed that KIM-1 is also upregulated in human brain death donors compared to living kidney donors[38]. Therefore, we concluded that KIM-1 measured at the time of brain death diagnosis is an independent predictive factor for short time kidney function after transplantation.

Other candidate markers such as the molecules CCL19/21 and PSMB8/9/10, were found by Kotsch *et al.*[24] and tested for posttransplantation clinical outcomes showing the potential to predict the development of DGF, acute rejection, and renal function after 6 months.

In addition, our group was able to demonstrate that elevated Ang2 levels in the brain death donor were associated with an increased risk of rejection and the decrease in the Ang1/Ang2 ratio with an increased risk of delayed graft function (unpublished). Further study is now required to confirm the clinical validity of serum Ang2 and to assess if angiopoietins are promising markers indeed to predict the quality of the renal graft.

CONCLUSION

To date, there is substantial evidence that brain death is associated with a cascade of hemodynamic, inflammatory, and immunologic events that affect the outcome of transplanted kidneys. Time has come to now focus not only on the victim of injury, for example the kidney graft in its recipient, but also on the source of the syndrome: the brain dead donor himself. Exact mechanisms and pathways responsible for the brain death induced renal injury are still largely unknown. So, although there is no doubt that further research is required to elucidate the mechanisms, we are now already in the position to further therapeutic interventions and introduce efficient biomarkers for brain death induced renal injury in a clinical setting. As the deceased donor shortage will prevail and more suboptimal donor organs accepted, we ought to focus on innovative methods to repair those organs that were consented by donors to benefit transplant recipients and we have in our custody for only a short while.

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CHAPTER 8

Modulation of the angiopoietin/ Tie2-system does not prevent brain death induced kidney injury

WH Westendorp
ZJ Veldhuis
PJ Ottens
RM Jongman
J Wiersema-Buist
H van Goor
M van Meurs
HGD Leuvenink

ABSTRACT

Background

Organs derived from deceased brain dead (DBD) donors show worse function than those derived from living donors, possibly due to the brain dead-induced inflammatory response. Our study aimed to evaluate if modulating the Ang/Tie2-system in favor of angiopoietin-1 (Ang1) and inhibiting angiopoietin-2 (Ang2) would lead to a decrease in brain death-induced inflammation in brain dead (BD) rats.

Methods

In an experimental model, rats (n=7 per group) were either exposed to 4hr of brain death or a sham-operation. BD was induced in anesthetized and ventilated rats by inflating a subdurally placed balloon catheter. rhAng1 or the synthethic Tie2 receptor agonist Vasculotide (VT) as well as the Ang2 inhibiting peptibody AMG386, or vehicle were administered 30 min prior to BD induction. After four hours of BD, serum, kidney and lung tissues samples were collected and stored. Routine biochemistry, RT-qPCR, and immunohistochemistry were performed.

Results

Plasma creatinine levels were higher in BD rats compared to the sham-operated rats. Plasma injury markers were increased after BD induction compared to the sham-operated rats ($p<0.001$). A clear increase in influx of PMNs was observed in renal tissue in BD animals. rhAng1, VT and AMG386 treatment did not show a protective effect on functional plasma levels or renal mRNA expression of injury markers when compared to controls. Tie2 mRNA expression was however significantly decreased in BD rats ($p=0.01$), which was not affected by rhAng1, VT or AMG386 treatment.

Conclusion

This rat study failed to demonstrate a beneficial effect of rhAng1, VT or AMG386 in experimental BD. A remarkable down regulation of renal Tie2 was observed in the BD rats which justifies further studying the Ang/Tie2-mechanisms and Tie2 preserving strategies in BD.

INTRODUCTION

In patients with end-stage renal disease (ESRD) renal transplantation is the preferred replacement therapy as it is associated with better outcomes and results in superior quality of life and reduced mortality compared to dialysis[1-3]. In renal transplantation, grafts are retrieved from living, deceased brain death (DBD) and deceased cardiac death (DCD) donors. Worldwide most donor kidneys are derived from DBD donors. Although mechanistically not fully elucidated, it is commonly accepted that the pathophysiological processes initiated by brain death are responsible for the inferior quality and survival of these donor kidneys compared to those from living related donors[4,5]. Experimental and clinical studies have shown that brain death (BD) causes hemodynamic instability, hormonal dysregulation, a marked inflammatory response and various other processes[6-8]. BD induces an immediate procoagulatory and pro-inflammatory activation of vascular endothelium together with inflammatory responses in potential donor organs[9-11]. Activated endothelium disrupts the constitutive Angiopoietin/Tie2-signaling, an important system in maintaining vascular quiescence[12].

Angiopoietins are natural ligands of the tyrosine kinase receptor Tie2 and are important mediators of angiogenesis and maintenance of vascular integrity[13]. Of the angiopoietin family, angiopoietin-1 (Ang1) to -4, Ang1 and Ang2 are best characterized[14,15]. Both angiopoietins bind Tie2 in a competitive matter with similar affinity[16,17]. The Tie2 receptor is primarily expressed on endothelial cells and early hematopoietic stem cells. Angiopoietin/Tie2-signaling cascades are next to angiogenesis involved in vascular stabilization and remodeling, as well as recruitment of pericytes and smooth muscle cells[14,18]. In healthy adults, Ang1 is expressed by pericytes and vascular smooth muscle cells at relatively constant levels and acts in a paracrine agonistic manner maintaining the Tie2 receptor in an activated state[19]. Ang1-mediated Tie2 phosphorylation provides an anti- apoptotic and anti-inflammatory signal to the endothelium, thereby leading to vessel stabilization and preventing vascular leakage[14,20-22]. Ang2 is highly produced and expressed by endothelial cells at sites of normal and pathological angiogenesis[23]. In contrast, Ang2 acts as an autocrine antagonist of Ang1-mediated Tie2 activation, competitively inhibiting Tie2 signal transduction[24,25]. Thereby, inflammatory responsiveness and vascular leakage increases and endothelial function impairs[16]. Ang2 can prime the endothelium to respond to cytokines and other inflammatory mediators[25-27]. Little is known about the exact mechanisms of Ang2 function on Tie2 and some studies presented Ang2 as a partial agonist, activating Tie2[16,18,23]. Endothelial storage granules, Weibel Palade bodies (WPB), store Ang2 and quickly release it into the systemic circulation upon pro-inflammatory stimulation[27-29]. It impairs endothelial function and increases inflammatory responsiveness and induces vascular leakage[30]. Thereby Ang2 primes the vascular endothelium to

respond to exogenous cytokines leading to vascular destabilization. Given the endothelial activation, inflammatory environment and hemodynamic instability in DBD donors, appropriate pretreatment of the DBD donor to counteract the endothelial destabilization by enhancing Ang1 mediated Tie2 phosphorylation or inhibiting the antagonistic properties of Ang2, may therefore be a tool to improve donor organ quality and subsequently transplant outcome.

In human sepsis, which has pathophysiological similarities to brain death, the Ang/Tie2- system has been shown to play a critical role[31]. Ang2 serum levels are increased up to 20fold during sepsis, which have also been associated with mortality[32]. Counteracting endothelial activation and the inflammatory response in the DBD donor by inhibiting Ang2 or enhancing Ang1-mediated Tie2 signaling may be of therapeutical value to improve donor organ quality and subsequently transplant outcome. In acute kidney and lung injury treatment with either acute administration of recombinant human Ang1 (rhAng1) or Ang1 gene transfer prevented capillary leakage and was shown protective[33-35]. Furthermore, Ang1 and an Ang1 variant prevented vascular permeability in experimental endotoxemia[36,37], improved survival in endotoxic shock[38]. In human sepsis the Ang/Tie2-system has also been shown to play a critical role[39] and in murine sepsis, administration of rhAng1 improved survival and sepsis- associated organ dysfunctions[33]. Vasculotide (VT), a synthetic Tie2 receptor agonist, counteracted microvascular endothelial dysfunction in murine abdominal sepsis[40]. The frequent occurrence of endotoxemia in brain death donors makes Ang1 an interesting candidate to improve graft quality in DBD donors as well[41]. Several preclinical and clinical studies have investigated anti-Ang2 therapy in the treatment of chronic rejection in rat cardiac allografts, malignant tumors including monoclonal antibodies and siRNA[42]. None of them is evaluated in the setting of the DBD donor.

Therefore, in this translational study we investigated whether modulating the Ang/Tie2- system in favor of Ang1 would exert protective effects on the kidney during BD. Furthermore, we explored if inhibiting Ang2-mediated Tie2 signaling and would diminish the brain death-induced endothelial activation and inflammatory response. We hypothesize that rhAng1, VT or the Ang2 inhibiting peptibody AMG386 could preserve renal graft quality and function in an experimental brain dead rat model.

METHODS

Animals

Adult male Fisher F344 rats weighing 250 - 300 g (Harlan, Horst, the Netherlands) were used in all experiments. For a subanalysis C57Bl/6 wildtype (WT), and C5aR$^{-/-}$ [43] and C5L2$^{-/-}$ [44] mice were used both on C57Bl/6 background, kindly provided by B. Lu, Harvard Medical School, Boston, USA. Both rats and mice were housed in a light- and temperature-controlled environment and had free access to food and water. Mice

were bred in the local animal facility in the University Medical Center Groningen. The studies were carried out under a protocol approved by the Institutional Animal Care Committee of the University of Groningen (project numbers 6259ABC and 6279AF). All animals received care in compliance with the guidelines of the local animal ethics committee according to Experiments on Animals Act (1996) issued by the Ministry of Public Health, Welfare and Sports of the Netherlands.

Study design

We used a two-step design to minimize the number of animals needed. Each experimental group consisted of 7 animals (table 1). In the first two experiments the aim was to increase the Ang1 availability and the third to inhibit the pro-inflammatory Ang2 properties (figure 1). In experiment 1, rats were randomly divided into four groups to study the effect of rhAng1 on BD and sham-operated animals. The number of animals was calculated using the method of Russ Lenth[45] with a meaningful difference of 50%, a variability (σ) of 0.3 and a power of 0.9. Sham-operated rats served as controls and were ventilated for half an hour under anesthesia before termination. This was in accordance with the requirement of the local Animal Welfare Committee guidance for the use of sham controls in experiments. BD rats were sacrificed 4 h after BD induction. rhAng1 (R&D systems, Minneapolis, USA), or saline was administered IV 30 minutes before the start of BD induction (experiment 1). In a subsequent experiment, Vasculotide (VT)[40], or PBS was administered IV 30 minutes before the start of BD induction (experiment 2). Dosages of rhAng1 (1 μg/kg) and VT (3 μg/kg) were based on previous experiments in rodents[40,46]. Vasculotide was provided by Sunnybrook Health Sciences Center, Toronto, Canada. In the third experiment, the anti-Ang2 antibody AMG386 (2.8 mg/kg subcutaneously, Amgen Inc., Thousand Oaks, USA) treatment was compared to saline treatment, both administered IV 30 minutes before the start of BD induction.

Table 1. Study design

Experiments	Group	Treatment	Number of animals
1	Sham-operated	Saline	7
	Sham-operated	1 μg/kg rhAng1 in saline	7
	Brain dead	Saline	7
	Brain dead	1 μg/kg rhAng1 in saline	7
2	Brain dead	PBS	7
	Brain dead	3 μg/kg VT in PBS	7
3	Brain dead	Saline	7
	Brain dead	2.8 mg/kg AMG386 in saline	7

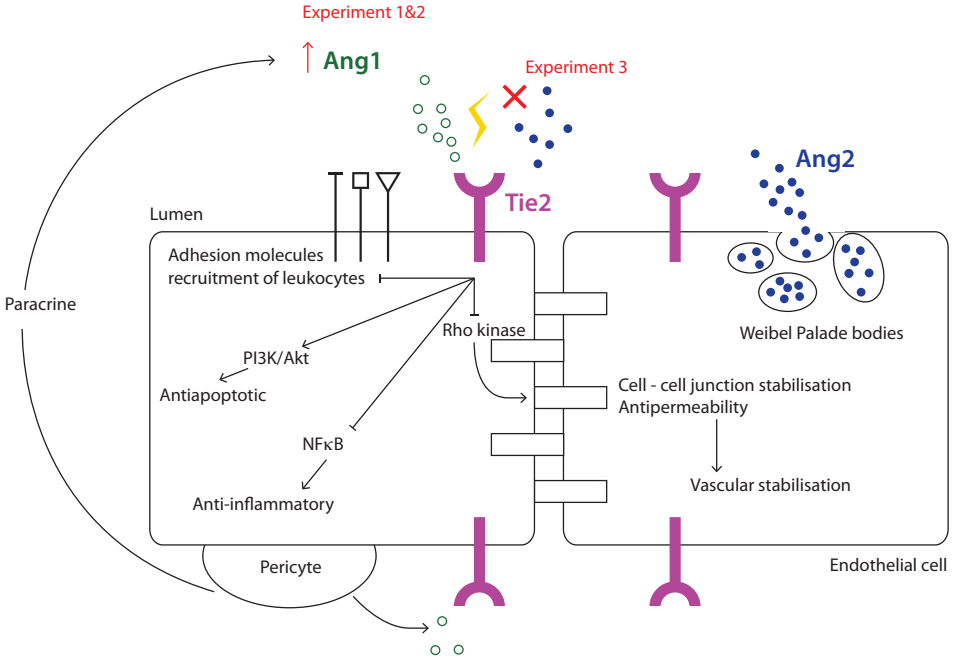

Figure 1. Overview of the Angiopoietin/Tie2-system at the endothelium. Adapted from van Meurs et al. Crit Care. 2009;13(2):207. Bench-to-bedside review: Angiopoietin signalling in critical illness - a future target? Experiment 1 & 2 were designed to increase Ang1 availability via rhAng1 and Vasculotide. In experiment 3 anti-Ang2 therapy of AMG386 was tested. Abbreviations: Ang1: angiopoietin-1, Ang2: angiopoietin-2, Tie2: tyrosine kinase receptor Tie2, NFκB: nuclear factor kappa- light-chain-enhancer of activated B cells.

Brain death induction

Brain death (BD) was induced as follows: animals were anesthetized using 5% isoflurane with 100% O_2. Cannulas were inserted in the femoral artery and vein for continuous mean arterial pressure (MAP) monitoring and fluid administration. Animals were intubated via a tracheostomy and ventilated throughout the experiment. A no. 4 Fogarty catheter (Edwards Lifesciences Co, Irvine, CA) was placed in the epidural space through a frontolateral burr hole, and slowly inflated (0.16 ml/min) with saline using a syringe pump (Terufusion, Termo Co, Tokyo, Japan). Inflation of the balloon was terminated once the MAP increased rapidly again after a characteristic period of hypotension, reflecting the autonomic storm. BD was confirmed by the absence of corneal and pupillary reflexes and a positive apnea test. Following confirmation of BD, anesthesia was terminated but ventilation continued. MAPs were maintained above 80mmHg using Hydroxyethyl starch (HAES) 10% (Fresenius Kabi AG, Bad Homburg, Germany) with a maximum rate of 1 ml/hr. If HAES was insufficient to maintain the MAP, noradrenaline 0.01 mg/ml was administered. To

maintain body temperature of the rats, a homeothermic blanket control system was used throughout the BD maintenance period. To achieve full muscle relaxation for abdominal surgery, rocuroniumbromide (0.6 mg/kg) was administered 15 minutes before the end of the brain death period of four hours. Five minutes before ending the brain death period, rats were heparinized with 500 IU heparin. A laparotomy was subsequently performed and blood was collected from the aorta. Organs were flushed with 0.9% saline and snap frozen in liquid nitrogen, another part was fixated in 4% paraformaldehyde. Collected plasma was stored at −80°C.

Plasma measurements

Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine and urea were measured in a routine fashion by the biochemistry lab of the University Medical Center Groningen (Mega, Merck, USA). Plasma levels of Ang2, Tie2 and IL-6 were determined by rat enzyme linked immonusorbent assays (ELISA) according to manufacturers' instructions (R&D Systems, Minneapolis, USA). All samples were analyzed in duplicate and read at 450 nm using a microplate spectrophotometer (Victor3, 1420 multi-label counter, Perkin Elmer, USA).

RNA isolation and cDNA synthesis

Total RNA was isolated from whole kidneys sections by using TRIzol (Life Technologies, Gaithersburg, MD). RNA samples were verified for absence of genomic DNA contamination by performing RT-PCR reactions in which the addition of reverse transcriptase was omitted, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. For cDNA synthesis, 1 μl T11VN Oligo-dT (0.5 μg/μl) and 1 μg mRNA were incubated for 10 min at 70°C and cooled directly after that. cDNA was synthesized by adding a mixture containing 0.5 μl RnaseOUT Ribonuclease inhibitor (Invitrogen, Carlsbad, USA), 0.5 μl RNase water (Promega), 4 μl 5 x first strand buffer (Invitrogen), 2 μl DTT(Invitrogen), 1 μl dNTPO's and 1 μlM-MLV reverse transcriptase (Invitrogen, 200U). The mixture was held at 37°C for 50 min. Next, reverse-transcriptase was inactivated by incubating the mixture for 15 min at 70°C. Samples were stored at −20°C.

Real-Time PCR on renal tissue

Fragments of several genes were amplified with the primer sets outlined in table 2. Pooled cDNA obtained from brain-dead rats were used as internal references. Gene expression was normalized with the mean of β-actin mRNA content. For each gene the expression was normalized relative to the mean CT value of the β-actin gene, using the β-actin signal from the same cDNA. Real-Time PCR was carried out in reaction volumes of 15 μl containing 10 μl of SYBR Green mastermix (Applied Biosystems, Foster City, USA), 0.4 μl of each primer (50 μM), 4.2 μl of nuclease free

Table 2. Primer sequences used

Gene	Primer sequences	Accession number	Amplicon size (bp)
β-actin	5′-GGAAATCGTGCGTGACATTAAA-3′ 5′-GCGGCAGTGGCCATCTC-3′	NM_031144.2	74
Angiopoietin-1	5′-TTGGGAATCCCTCTGGTGAAT-3′ 5′-CTGCCTCTGACTGGTTATTGCA-3′	NM_053546.1	67
Angiopoietin-2	5′-TATGAGAAGGCCATGGTTAGAATC-3′ 5′-CGTAACATTCTTGCTTGTGACAGA-3′	XM_344544.3	70
Tie2	5′-AAGAGCAGCAGGGAAGAGGG-3′ 5′-TGGAATGATTTGGATGCTGTAGAA-3′	NM_001105737.1	71
IL-6	5′-CCAACTTCCAATGCTCTCCTAATG-3′ 5′-TTCAAGTGCTTTCAAGAGTTGGAT-3′	NM_012589	89
TNF-α	5′-AGGCTGTCGCTACATCACTGAA-3′ 5′-TGACCCGTAGGGCGATTACA-3′	NM_012675.2	67
KIM-1	5′-AGAGAGAGCAGGACACAGGCTTT-3′ 5′-ACCCGTGGTAGTCCCAAACA-3′	NM_173149.1	75
ICAM-1	5′-CCAGACCCTGGAGATGGAGAA-3′ 5′-AAGCGTCGTTTGTGATCCTCC-3′	NM_012967.1	251
HO-1	5′-CTCGCATGAACACTCTGGAGAT-3′ 5′-GCAGGAAGGCGGTCTTAGC-3′	NM_012580.2	74

water and 10 ng of cDNA. All samples were analyzed in triplicate. Thermal cycling was performed on the Taqman Applied Biosystems 7900HT Real Time PCR System with a hot start for 2 min at 50°C followed by 10 min 95°C. Second stage was started with 15 s at 95°C (denaturation step) and 60s at 60°C (annealing step and DNA synthesis). The latter stage was repeated 40 times. Stage 3 was included to detect formation of primer dimers (melting curve) and begins with 15s at 95°C followed by 60 s at 60°C and 15 s at 95°C. Primers were designed with Primer Express software (Applied Biosystems, Foster City, USA) and primer efficiencies were tested by a standard curve for the primer pair resulting from the amplification of serially diluted cDNA samples (10 ng, 5 ng, 2.5 ng, 1.25 ng and 0.625 ng) obtained from brain-dead rats. Specificity of qPCR products was routinely assessed by performing a dissociation curve at the end of the amplification program and by gel electrophoresis on a 1.5% agarose gel. PCR efficiency was found to be $1.8 < \epsilon < 2.0$. Gene expression was normalized with the mean of β-actin mRNA content and calculated relative to controls using the relative standard curve method. Results were finally expressed as $2^{-\Delta\Delta CT}$ (CT: Threshold Cycle), which is an index of the relative amount of mRNA expressed in each tissue.

HIS-48 staining on renal tissue cryosections

The polymorphonuclear cell (PMN) count was used as a marker of inflammation. To detect PMNs in the kidney, immunohistochemistry was performed on 5 µm renal tissue

cryosections of experiment 1. Sections were fixated for 10 minutes using acetone. Next, sections were stained with HIS-48 mAb (supernatant, two times diluted) using an indirect immunoperoxidase technique. Endogenous peroxidase was blocked using H_2O_2 0.01% in PBS for 30 minutes. After thorough washing, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG as a secondary antibody for 30 minutes followed by goat anti-rabbit IgG as a tertiary antibody for 30 minutes (both from Dako, Glostrup, Denmark). The reaction was developed using 9-amino- ethylcarbazole as chromogen and H_2O_2 as substrate. Sections were counterstained using Mayers' hematoxylin solution (Merck, Darmstadt, Germany). Negative antibody controls were performed. Localization of immunohistochemical staining was assessed by light microscopy. For each tissue section, positive cells were counted in 10 microscopic cortical fields of the glomerulus at 20x magnification. Results were presented as number of positive cells per cortical area (μm^2).

Quantification of Tie2 protein by Western Blot analysis

To gain further insight in the Tie2 availability on protein level, we tried several techniques such as an ELISA and Western Blot (WB) analysis total Tie2. Unfortunately we experienced technical difficulties using the rat kidneys of our above-mentioned experiments leading to irreproducible, invalid results. In order to give an approximation of the Tie2 protein level in brain death compared to controls, we used BD and sham-operated mouse kidneys of another experiment. In brief, C57Bl/6 male mice (wildtype, $C5aR^{-/-}$, and $C5L2^{-/-}$) aged 8 to 12 weeks, with a weight of 25-28 grams were used. Per mouse strain, 8 animals were subjected to 3h of brain death as described above. During brain death, a MAP above 60 mmHg was considered to be normotensive. To maintain stable blood pressure, 50 µl saline with lepirudin was administered every 15 minutes via the jugular vein cannula. When blood pressure dropped below 60 mmHg, an additional 50 µl of saline was administered. A maximum of 1200 µl of saline was administered during the 3 hours brain death period. 30 minutes after declaration of brain death, ventilation was switched to a mixture of oxygen and medical air (50%-50%). Body temperature was continuously monitored and maintained at 37°C as described above. At time of sacrifice, blood and kidneys were collected for analysis. For total Tie2 WB analysis, kidneys of two experimental groups (sham+saline, n=3 and BD+saline, n=3) were homogenized in RIPA buffer (150mM NaCl, 50mM Tris pH 8.0, 0,5% Na-deoxycholate, 0,1% SDS, 1% IGEPAL) supplemented with protease and phosphatase inhibitors (Roche, Almere, The Netherlands) and 1 mM Na_3VO_4. Protein concentrations were determined by DC Protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

For statistical analyses of more than two groups, the Kruskal-Wallis test was performed, followed by the Mann-Whitney posttest. For comparison of two

groups, a Mann-Whitney test was performed. Results are presented as mean ± SEM (standard error of the mean). All the statistical tests were 2-tailed with $p<0.05$ regarded as significant. Analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, USA).

RESULTS

Brain death experiments

Induction of BD showed a consistent and uniform pattern in MAP alterations as described before[47] and took approximately 30 minutes after commencing balloon inflation. During the experiments, all animals were successfully kept at a MAP of at least 80 mmHg after the confirmation of BD. In experiment 1, saline treated animals required 4.1 ml ± 1.1 infusion of HAES to maintain MAP whereas in the rhAng1 treated group 3.7 ml ± 0.3 was needed (NS). Additional hemodynamic support by administration of noradrenaline also not differed between the groups (1.2 ml ± 0.6 in the saline treated group vs. 1.2 ml ± 0.3 in the rhAng1 treated group). In experiment 2, in both the PBS and VT treated group 1.4 ml ± 0.5 infusion of HAES was needed. In the PBS treated group, 1.8 ml ± 1.3 of noradrenaline vs. 2.3 ml ± 2 in the VT treated group was needed. In the saline treated group of experiment 3 infusion of 1.1 ml ± 0.7 HAES infusion was needed to maintain MAP whereas in the AMG386 treated group 1.2 ml ± 0.5 was required. Administration of noradrenaline was 0.9 ml ± 0.9 in the saline treated group vs. 1.7 ml ± 1.6 in the AMG386 treated group.

Plasma biochemistry after brain death

Plasma levels of kidney function and injury markers are demonstrated in figure 2, (experiment 1), 3 (experiment 2) and 4 (experiment 3). Plasma creatinine levels were significantly higher in BD animals treated with saline (96±8.6 μmol/L) compared to 35±2.7 μmol/L in saline treated sham-operated animals ($p=0.0006$). Creatinine levels in BD animals treated with rhAng1 or VT did not significantly differ from BD animals treated with a vehicle.

Plasma ALT, AST and LDH were significantly increased after BD induction and saline treatment compared to saline treated sham-operated animals (ALT: 113±8.7 vs. 55±2.5 U/l, $p=0.0006$; AST: 160±18.7 vs. 73±3.7 U/l, $p=0.006$; LDH: 366±77.8 vs. 194±29.2 U/l, $p=0.04$). Plasma Ang2 and IL-6 were significantly increased in the saline treated BD group compared to the sham-operated animals treated with saline (Ang2: 6878±556 vs. 4387±273 pg/ml, $p=0.006$; IL-6: 71±21.8 ng/ml vs. undetectable). Treatment with rhAng1 or VT did not significantly affect these plasma levels compared to their controls. In the third experiment, AMG386 treatment did not affect plasma creatinine levels (saline: 75.5±5.1 μM vs AMG386: 72.5±2.2 μM). Also, ALT, AST and LDH levels did not differ between treatment

and controls. Plasma IL-6 and Ang2 were increased in the AMG386-treated group (saline: 197.2±53.8 vs AMG386 392.1±60.1 pg/ml, p=0.03 and saline: 5621±599.7 vs AMG386:20234±375.1 pg/ml, p<0.001 respectively).

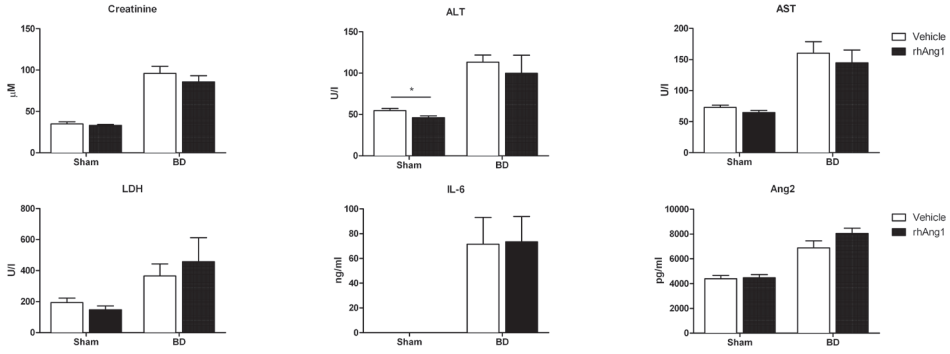

Figure 2. Plasma levels of renal function and cellular injury markers of experiment 1, administering rhAng1 (1 µg/kg) to sham-operated and BD rats. Data of 7 animals are presented as mean±SEM after 4h of brain death. Sham-operated animals and/or treatment with a vehicle, saline, represent the controls. Plasma levels were not affected by rhAng1 treatment (p>0.05).

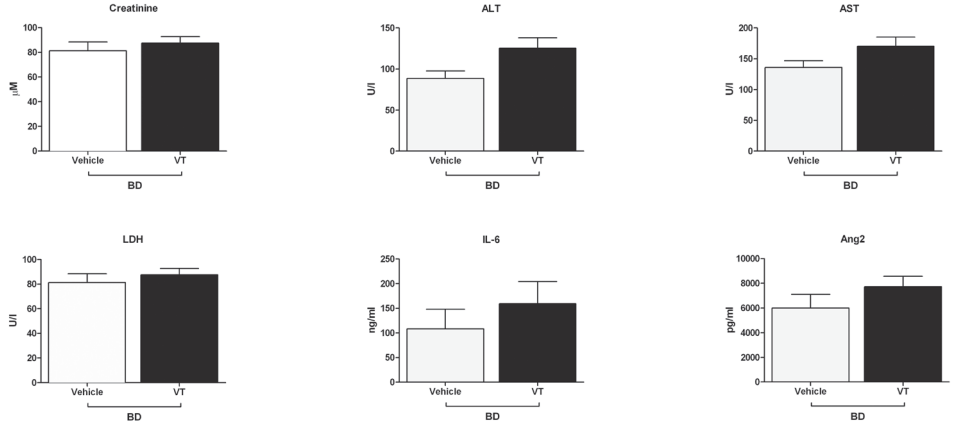

Figure 3. Plasma levels of renal function and cellular injury markers of experiment 2, administering VT (3 µg/kg) to BD rats. Data of 7 animals are presented as mean±SEM after 4h of brain death. BD animals treated with a vehicle, PBS, represent the controls. Plasma levels were not affected by VT treatment (p>0.05).

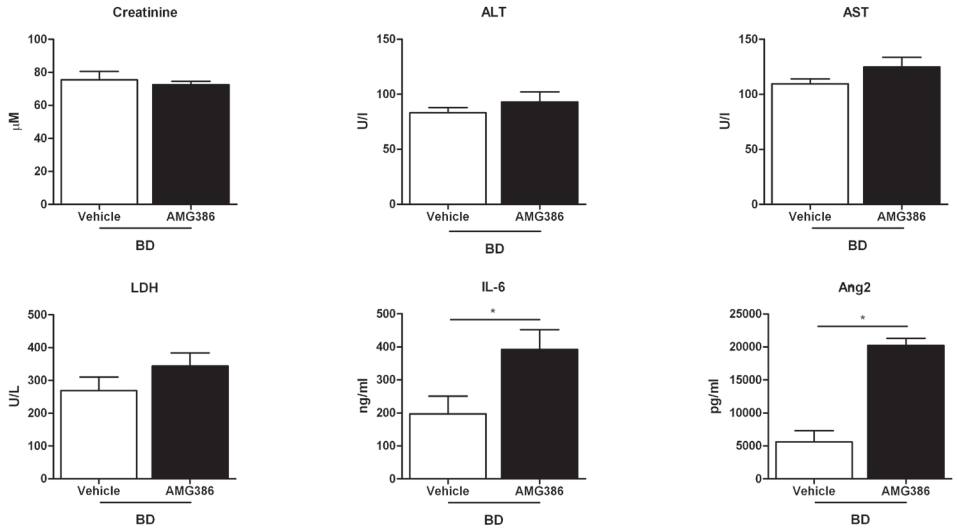

Figure 4. Plasma levels of renal function and cellular injury markers of experiment 3, administering anti-Ang2 antibody AMG386 (2.8 mg/kg) to BD rats. Data of 7 animals are presented as mean±SEM after 4h of brain death. BD animals treated with a vehicle, saline, represent the controls. Plasma levels of IL-6 and Ang2 were increased in the AMG386-treated group ($p<0.001$).

Renal gene expression

As demonstrated in figure 5, quantitative RT-PCR of experiment 1 showed significantly increased gene expression of TNF-α, IL-6, KIM-1, HO-1 and ICAM-1 in kidneys as a consequence from BD treated with saline compared to sham-operated controls treated with saline (TNF-α: 2.3±0.5 vs. 1±0.2, IL-6: 8.7±2.2 vs. 0.02±0.007, KIM-1: 16±3.4 vs. 0.04±0.009, HO-1: 5.2±1.0 vs. 0.05±0.006, ICAM-1: 0.70±0.2 vs. 0.07±0.04, $p<0.05$). In the sham-operated animals, renal gene expression of TNF-α was down-regulated by rhAng1 treatment compared to saline (0.38±0.08 vs. 1±0.2, $p=0.03$). Relative renal gene expression of Ang1 and Ang2 did neither significantly differ between BD and sham-operated animals nor between saline and rhAng1 treated animals. Tie2 gene expression was significantly decreased in BD induced animals compared to sham-operated animals. rhAng1 administration did not influence Tie2 gene expression. In the second experiment administrating VT or PBS to BD induced rats, renal ICAM-1, IL-6 and Tie gene expression were measured (figure 6). Their gene expression was not affected by VT treatment. Quantitative RT-PCR of experiment 3 did not show a significant different gene expression of Ang1, Ang2, Tie2, IL-6 or ICAM-1 in kidneys as a consequence from BD rats treated with AMG386 compared to controls (figure 7).

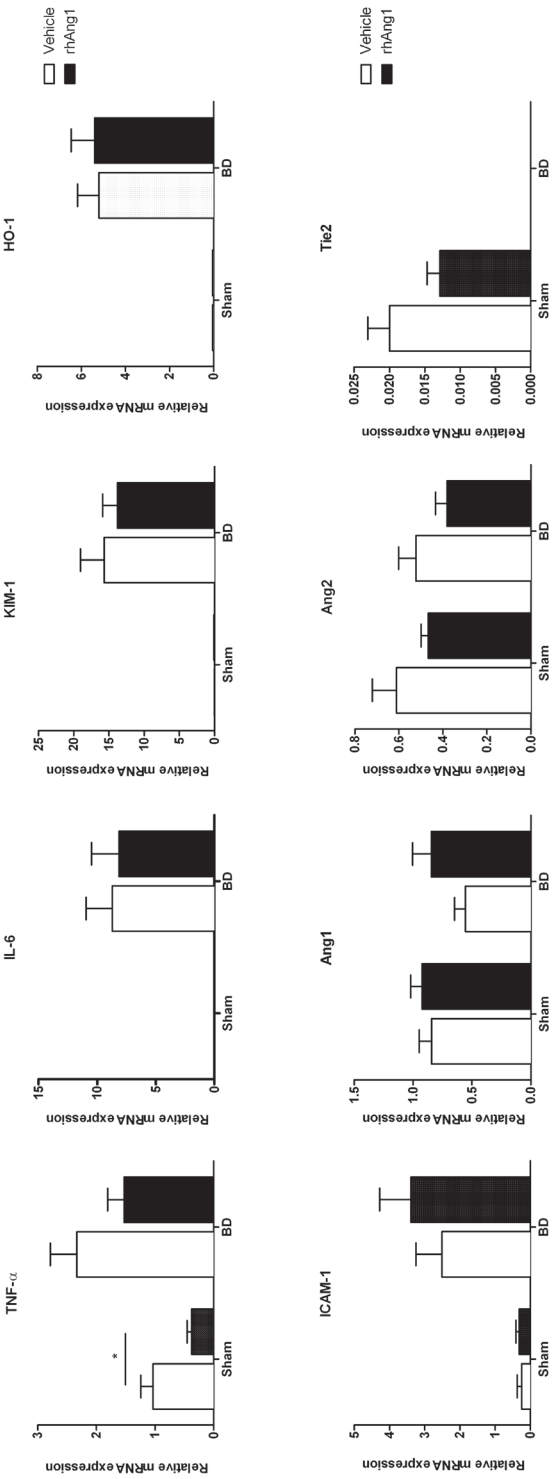

Figure 5. Relative renal gene expression of experiment 1 administering rhAng1 (1 µg/kg) to sham-operated and BD rats. Renal mRNA levels of 7 animals per group presented as mean±SEM after 4 h of brain death. TNF-α decreased in the rhAng1 treated sham-operated animals compared to the saline treated sham-operated animals (p=0.03). Relative expression of all genes was increased after BD induction (p<0.05) except for Tie2, which was markedly reduced (p<0.05). mRNA levels were normalized to the β-actin signal of the same cDNA.

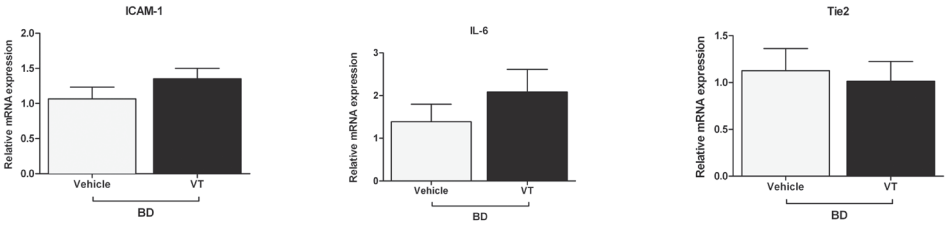

Figure 6. Relative renal gene expression of experiment 2 administering VT (3 µg/kg) to BD rats. Renal mRNA levels of 7 animals per group presented as mean±SEM after 4h of brain death. Renal ICAM-1, IL-6 and Tie2 mRNA expression was not affected by VT treatment in BD rats ($p>0.05$). mRNA levels were normalized to the β-actin signal of the same cDNA.

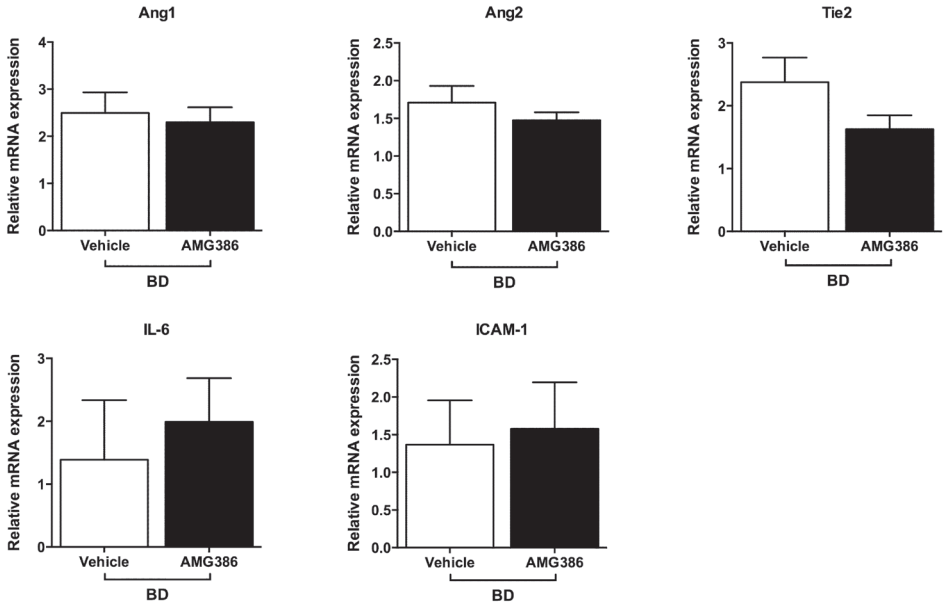

Figure 7. Relative renal gene expression of experiment 3, administering anti-Ang2 antibody AMG386 (2.8 mg/kg) to BD rats. Renal mRNA levels of 7 animals per group presented as mean±SEM after 4h of brain death. Renal Ang1, Ang2, Tie2, IL-6 and ICAM-1 mRNA expression was not affected by AMG386 treatment in BD rats ($p>0.05$). mRNA levels were normalized to the β-actin signal of the same cDNA.

Renal PMN infiltration

Renal cryosections of experiment 1 were stained with HIS-48 mAb to study the influx of PMNs (figure 8). The number of positive cells per cortical area in the glomerulus was 0.6±0.2 in sham-operated controls and increased significantly to 6±1.2 in the BD+saline group ($p=0.004$).

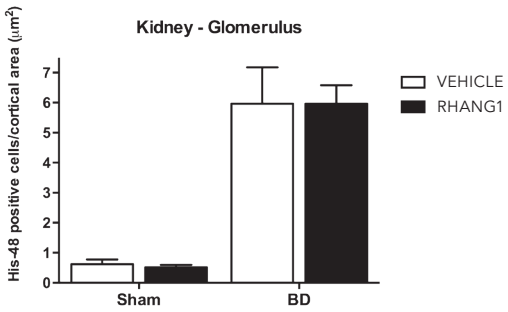

Figure 8. Renal PMN infiltration, quantification and HIS-48 staining of experiment 1 administering 1 μg/kg rhAng1 to sham-operated (n=7) and BD rats (n=7). Quantification of PMN infiltration in cortical glomeruli of renal cryosections. PMN infiltration was increased after BD induction, but not affected by rhAng1 treatment (p>0.05).

Quantification of Tie2 protein

Renal Tie2 protein quantification of a mouse BD experiment is demonstrated in figure 9. The renal Tie2/β-actin ratio was not significantly different between sham- and BD operated rats (0.21±0.06 vs. 0.11±0.03, p=0.2).

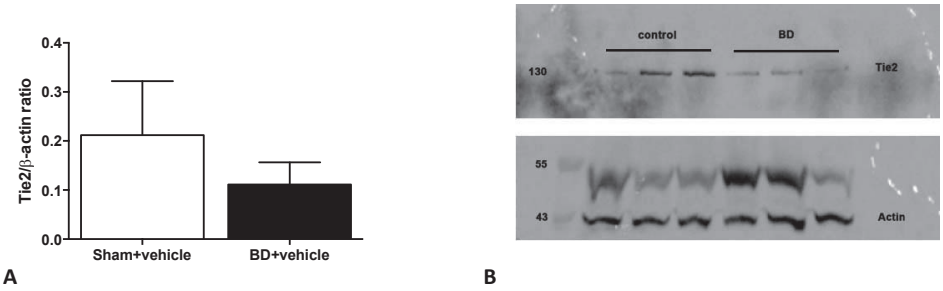

Figure 9. Relative Tie2 protein quantification of sham-operated and BD mice. A) Renal total Tie2 protein expression of 3 mice per group after 4h of brain death presented as mean±SD, normalized to the β-actin signal. BD did not significantly alter the protein expression of total Tie2 in the mouse kidney. B) 100 μg protein input of mouse kidney was used for WB analysis. β-actin was used as a loading control.

DISCUSSION

To our knowledge, this is the first study investigating Ang/Tie2-axis intervention in experimental brain death. We aimed to modify the Ang1 levels in our BD rat model by administrating rhAng1 and the synthetic drug-like Tie2 agonist, VT. In the same model we studied administration of AMG386, an Ang2 inhibiting peptibody. Besides the finding that exogenous treatment with rhAng1, VT or

AMG386 did not attenuate brain death-induced inflammation nor improve renal function a major finding of this study is the remarkable decrease of Tie2 gene expression in the kidney after BD.

In the literature, no other (pre)clinical studies regarding donor pretreatment via Ang1 have been reported. Both rhAng1 and VT have been studied in various preclinical models including murine sepsis, which has pathophysiological similarities to brain death. Intravenous rhAng1 treatment showed significant improvement of sepsis-associated organ dysfunctions and survival time, possibly via preserving the endothelium[33]. In an abdominal sepsis model in mice, systemic VT administration protected against sepsis-induced endothelial barrier dysfunction and reduced mortality[40]. Inhibiting Ang2 gained more and more attention in preclinical studies, also outside anti angiogenesis therapy in tumors. Various studies have shown that it is possible to inhibit Ang2-induced Tie2 phosphorylation by antibodies in pre-clinical studies[42,48-51]. One study showed that Ang2 inhibition decreased the expression of adhesion molecules in an ischemic mouse hind limb model[52]. A peptibody, inhibiting the interaction between the Tie2 receptor and Ang1 and Ang2 was the first to enter a phase III clinical trial demonstrating promising results[53]. Although the strategy to inhibit Ang2 is rather novel in the field of transplantation, another anti-Ang2 antibody demonstrated promising results in rat cardiac allografts after brain death. *Ex vivo* treatment of the allografts with anti-Ang2 antibody reduced endothelial cell adhesion molecule expression, leukocyte infiltration and the activation of the innate immune response[49].

The current study was designed as a proof of principle study to evaluate the effect of modulating the Ang/Tie2-system in favor of Ang1, by enhancing Ang1 availability or inhibiting Ang2, on the pro-inflammatory response caused by BD. We expected a rapid brain death-induced endothelial activation quickly triggering the WPB Ang2 release since previous studies using our BD rat model showed increased expression of endothelial adhesion molecules, endotoxins and cytokine levels such as IL-6 at 4 hours of BD compared to sham- operated rats[47,54-57]. Furthermore, including profound ICAM-1, HO-1, TNF-α, KIM-1 mRNA expression and renal influx of PMNs were shown to indicate BD-induced inflammation[54,55,57,58]. In another BD rat model it was demonstrated that circulatory inflammatory molecules influence the state of peripheral organs in which expression of lymphocyte- and macrophage associated products were increased[6]. In line with previous studies using an identical experimental brain death model, we found increased functional and inflammatory markers in the BD animals compared to sham-operated controls[6,59-61]. Unfortunately, no beneficial effects of rhAng1, VT or AMG386 donor pretreatment on these injury parameters were found.

Our study did not show increased Ang1 mRNA expression in the rhAng1 or VT treated groups indicating no feed-back on the native Ang1 release. This could

be explained by the used low dosages (1 µg/kg and 3 µg/kg respectively), which were based on previous sepsis experiments in mice. We can therefore not exclude a potential anti-inflammatory effect when higher dosages are used. However an alternative and more eligible explanation may be the marked decrease in Tie2 mRNA expression, also suggested by the trend in reduced protein levels via Western Blot analysis, which we observed in all BD groups. Under normal conditions, Tie2-phosphorylation mediated by Ang1 prevents apoptosis[20] and leads to anti-inflammatory survival signaling, stabilizing the endothelium[14,22]. However if Tie2 expression is diminished due to BD induction, Ang1-mediated Tie2 phosphorylation may be futile regardless of the availability of endogenous or exogenous Ang1. As demonstrated by *in vitro* experiments, Tie2 expression can be regulated by its ligands Ang1 and Ang2[18,62]. One could speculate that in response to rhAng1 or VT, Tie2 is rapidly internalized and targeted for degradation and thereby diminishing the ability of the cell to respond to further stimulation[18]. Receptor internalization and degradation is considered to be a mechanism attenuating downstream signal transduction resulting in an overall loss of receptors from the cell surface, thereby diminishing the ability of the cell to respond to further stimulation[63,64]. In human umbilical vein endothelial cells (HUVECs), Tie2 was rapidly internalized and targeted for degradation in response to Ang1[18]. Therefore, exogenous Ang1 treatment may not be the preferential route to manipulate the Ang/Tie2-system in BD. To date, the mechanism regulating Tie2 internalization is unknown. Another explanation for the absence of an effect of rhAng1 or VT may be the capability of Ang1 to bind to other surface receptors such as Tie1 and integrins[65,66]. In that way, the observed downregulation of Tie2 mRNA in BD may be caused by BD related cascades independent of exogenous Ang1. However, as Ang1 and Ang2 are released into the medium after binding to endothelial cells[18], they are capable of rebinding Tie2 on fresh endothelial cells.

The increased plasma Ang2 we found in the AMG386-treated animals compared to the saline-treated controls in the third experiment may be caused by the used dosage. Ang2 has been characterized as a context-dependent antagonist since in some studies Tie2 binding by high Ang2 levels resulted in receptor activation with similar outcomes to Ang1[67,68]. Since we measured increased Ang2 levels in the AMG386 treated group, one could speculate that Ang2 could thereby act as a Tie2 agonist, inducing anti-inflammatory and anti-apoptotic survival signals. Except looking at the increased plasma IL-6 mRNA expression, that could hardly have been the effect. This upregulation is more likely caused by BD-induced inflammation itself[69] with IL-6 playing an important role in activating the inflammatory response what presumably caused enhanced WPB exocytosis of Ang2. Regardless of the pathophysiolocal effects caused by BD, there seems to be a role for the Ang/Tie2-system in renal failure and repair. Ang2 over-expression in mice causes proteinuria and apoptosis of glomerular endothelial cells[70]. In a rat

model of glomerulonephritis, Ang1 and Ang2 are overexpressed by podocytes and Tie2 is overexpressed by endothelial cells, all in a time- dependent manner during the repair phase[71].

Despite efforts made, several questions remain unsolved. In this study due to the absence of an appropriate rat Ang1 ELISA, no definite conclusions on available systemic Ang1 can be made. Next, we were unable to determine the phosphorylated and systemic Tie2 availability due to the absence of a working method in our lab. Several anti-bodies and methods were tested, unfortunately without satisfactory results. Also we do not know how quickly Tie2 expression is affected in the BD setting since we have not evaluated the effect of BD on Tie2 expression at earlier time points after BD induction. Despite these limitations, our data reveal a remarkable effect of BD on Tie2 mRNA expression and protein level which underlines the suggestion of the Ang/Tie2-axis playing a role in BD. This finding is to some extent with in line with the reported decreased Tie2 expression in post-mortem renal biopsies of patients with sepsis[72]. As the pathophysiology of sepsis and BD are alike, perhaps Tie2 preserving strategies, may be another therapeutical target in BD.

In conclusion, this rat study was not able to show any beneficial effect of rhAng1, VT or AMG386 in terms of improved renal function, lower inflammation, enhanced Ang1 or decreased Ang2 expression in experimental brain death. However based on the remarkable down regulation of Tie2 we feel that further studying the mechanism of the Ang/Tie2-system in BD is justified. Better mechanistic understanding of the Ang2/Tie system is warranted before translation of this endothelial signaling system as a therapeutic target in human BD studies is feasible.

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CHAPTER 9

Summary, discussion and future perspectives

In patients with end-stage renal disease (ESRD), renal transplantation is the preferred therapy with enhanced patient survival compared to dialysis therapy. In renal transplantation, kidneys derived from living donors have better graft survival compared to kidneys obtained from deceased donors[1,2]. In order to completely utilize the donor pool, interventions improving transplant outcome, for instance by diminishing brain death-induced inflammation, are warranted. The demonstrated role of the endothelial Ang/Tie2-system in sepsis and endotoxemia made us question how this system would relate in the deceased brain death donor and subsequently other aspects of renal transplantation. We hypothesized that the Ang/Tie2-system plays a critical role in endothelial activation before and throughout renal transplantation, thereby affecting donor organ quality and patient and allograft outcome.

To study whether changes in angiopoietin expression were already present in the pre-transplant setting, we first studied plasma Ang1 and Ang2 in diaysis patients. In **chapter 2**, we show Ang1 and Ang2 levels in a well-defined cohort of 100 patients on hemodialysis. A marked peak of Ang2 levels was seen in these patients after 60 minutes of hemodialysis. Furthermore, significant associations between Ang2 and markers of inflammation, fluid overload and cardiac damage were found. In contrast no association was found between Ang1 and clinical parameters or dialysis outcome. Prospective analysis revealed that Ang2 levels are associated with a higher incidence of all-cause mortality and cardiovascular events. This extends previous research on Ang2 in dialysis, demonstrating that angiopoietin levels correlated with the presence and severity of coronary heart disease and peripheral arterial disease. Since CKD patients and patients on HD are characterized by endothelial dysfunction and endothelial dysfunction itself is a known cause of Ang2 release by Weibel Palade bodies, we speculate that the elevated predialysis Ang2 levels reflect endothelial activation. This is substantiated by the strong association between Ang2 and pro-endothelin levels that we found in these patients. These results underline the importance of understanding the responsible mechanisms concerning the Ang/Tie2-system. Elucidating this will provide the fundament for therapeutic intervention studies on the Ang/Tie2-system in hemodialysis.

Although circulating angiopoietin levels have been studied in renal transplant recipients and small numbers of living and deceased kidney donors[3-6], little is known of the angiopoietin response in the complete renal transplantation cascade, from donor to its paired recipient. Since is has become evident that Ang2 is the most rapid responder to pathologic circumstances and the fact that it seems to play a predictive role in various conditions, we studied plasma levels and arteriovenous renal Ang2 release in living donor renal transplantation in **chapter 3**. Both in donors and recipients, plasma Ang2 changed during the operation compared to the preoperative levels while reperfusion did not affect renal Ang2 release. In contrast, another study on renal Ang2 release in living and DBD transplantation

demonstrated increased renal Ang2 release of the reperfused kidney grafts in living and DBD transplantation. The use of the same Ang2 ELISA in that study makes a technical cause of the difference in results between our studies unlikely. Perhaps both living donor populations are not comparable when it comes to the degree of endothelial activation before and during nephrectomy. Additional multi-center Ang2 analysis in renal transplantation are required to further shed light on the intracentral differences in living donor selection and endothelial activation during retrieval.

In **chapter 4** we describe Ang1 and Ang2 levels in renal transplant recipients (RTR) and their controls. In line with previous studies by our group and others, Ang2 associated with markers of inflammation and cardiac damage. This is consistent with previous data in which Ang2 levels are positively associated with CRP in another cohort of RTR[4]. Our results showed higher plasma Ang2 in RTR who received a kidney from a deceased donor compared to those RTR who received a kidney from a living donor. In these deceased donor-RTR, Ang2 was associated with graft failure and mortality. Possibly RTR suffer a pre-inflammatory state which aggravates in case there is a risk for organ failure, reflected by increased Ang2-supported endothelial activation[7-12,15,16].

Deceased brain dead (DBD) donors traditionally have been the main source of donor organs in renal transplantation. The results of further studying the histopathological characteristics of the morphological inflammatory responses caused by brain death in (pre-) transplant renal biopsies are described in **chapter 5**. Although our group and others have demonstrated the detrimental effect of BD on organ quality and graft survival, it is still difficult to predict donor organ quality at time of donor management and evaluation. Early pre-transplant predictions of transplant outcome by numerous histopathological damage parameters and scoring systems have been studied. These results have however not led to a unanimous approach[13-16]. In addition to the classical damage parameters, we were particularly interested in lymph vessel density (LVD). Although the importance of LVD in the development and progression of renal pathophysiology -and transplantation has been convincingly shown, it is not well studied in pre-transplant biopsies and BD. As anticipated, pretransplant biopsies of DBD donors showed increased LVD and pro-fibrotic, vascular and inflammatory damage compared to the biopsies obtained from living donors. Within the DBD group, LVD was correlated with signs of arteriopathy such as hyalinosis and with markers of interstitial injury such as interstitial fibrosis. Whether the increase in LVD is the cause or the effect of vascular damage is unknown, but it might well be that pathologic thickening of the vascular wall hampers normal vascular function including fluid transport, thereby resulting in increments in LVD. Validation of LVD in the assessment of kidney quality before transplantation and evaluation whether a separate scoring approach is required for DBD donors needs to be determined in prospective analysis.

In **chapter 6** deceased donor and recipient polymorphisms in the Ang2 gene were determined in a large renal transplant cohort. Associations between Ang2 single nucleotide polymorphisms (SNPs) and the development of acute lung injury and increased risk of acute respiratory distress syndrome have been reported. These findings together with the possibility of changed Ang2 protein expression and effect among different Ang2 SNPs, and our suggested role of Ang2 in deceased donation and RTR, resulted in a genetic renal transplant cohort Ang2 study. Tagging Ang2 SNPs were associated with death censored graft survival when analyzed in both donor and recipient. No association was found with all-cause mortality. To confirm the role of Ang2, the functionality of these Ang2 SNPs has to be confirmed by replication studies.

In **chapter 7** we reviewed the literature on brain death (BD) and presented an overview of the current knowledge of renal injury caused by BD. We described the current knowledge during this unphysiological state, its effect on kidney organ quality, potential mechanisms of repair and its relevance for renal transplant outcome. Intracranial hemorrhage or traumatic brain injury are usually the cause of brain compression leading to irreversible cerebral injury. Ultimately the brain stem herniates with loss of brain stem reflexes, a Cushing reflex due to catecholamine release followed by hypotension and finally complete circulatory arrest. Due to complete dysfunction of the hypothalamo-pituitary axis most patients suffer from diabetes insipidus. Shortly after the onset of brain death, high levels of circulating proinflammatory cytokines such as interleukin-6 can be detected. BD results in endothelial activation and increased influx of polymorphonuclear neutrophils (PMNs) and macrophages in kidney, liver and intestine. The inflammatory response on organ level requires interventional strategies aiming at enhanced graft quality and subsequently better graft survival of kidneys obtained from DBD donors.

Frequent occurrence of endotoxemia has been shown in DBD donors[17]. Endotoxemia influences the Ang/Tie2-system by triggering an increased vascular permeability[18]. A disturbed Ang1/Ang2 ratio in favour of Ang2 is associated with increased mortality during sepsis, while a ratio in favour of Ang1 maintains vascular integrity and dampens the inflammatory response[19-21]. The known endotoxemic status together with the pathophysiological similarities between BD and sepsis makes the Ang/Tie2-system an interesting intervention target in aiming to counteract the detrimental consequences of BD. Experimental studies on recombinant and Ang1 derivates like recombinant human Ang1 (rhAng1) and Vasculotide (VT), have demonstrated promising results in murine sepsis and protecting adult vessels against plasma leakage[21-23]. Therefore, we studied Ang/Tie2-axis manipulation via two Ang1 enhancing strategies in our brain death rat model.

In **chapter 8** we describe the first proof of principle experiments aiming to modulate the Ang/Tie2-system by testing exogenous Ang1 administration and Ang2 inhibition in experimental brain death. BD rats were treated with

rhAng1 or a vehicle and compared with sham-operated rats. In a second experiment, BD rats were treated with VT or a vehicle. Unfortunately, neither rhAng1 nor VT treatment did protect against brain death induced inflammation in rat kidneys. This may be explained by a dosage or timing problem. Possibly Ang1 enhancing therapy may be more beneficial during BD itself, immediately competing with the increased Ang2 in order to bind Tie2. The dosages we used were based on previous sepsis experiments in mice. Even though we took the difference in metabolism and weight of the rats into account, the dosages may have been too low. We can therefore not exclude a potential anti-inflammatory effect when higher dosages are used. Real-time PCR analysis revealed a marked decrease in Tie2 mRNA expression in BD rats which could be another explanation why the Ang/Tie2-system was not beneficially affected by exogenous administration of these Ang1 derivates. Although our analysis made it indefinable whether the Ang/Tie2-system was actually modulated in favour of Ang1 by these dosages, the results on mRNA expression made this highly unlikely.

Although the majority of preclinical studies on similar pathophysiological conditions as BD have approached intervention strategies via the Ang/Tie2-system by upregulating Ang1, recent literature is more and more focussing on Ang2 as the most dynamic player in endothelium activated processes. Others have reported promising results on anti-Ang2 therapy in various preclinical models including a study on cardiac rat allografts. Therefore, in a third experiment we studied whether inhibiting Ang2 could attenuate BD induced renal inflammation in the rat model mimicking the DBD donor. This study was designed as a single dose pilot in which systemic Ang2 was not reduced by this treatment. We observed no reduction in systemic or renal inflammation in this group compared to the vehicle treated BD rats. Until now we and others have studied Ang/Tie2-intervention strategies approaching Ang1 or Ang2 levels. A dosing titrating study on exogenous Ang1 or an Ang2 inhibitor will learn us which dosage should be further studied. After this an experimental BD time course-effect study of Ang/Tie2-system intervention will give the opportunity to draw more definite conclusions. Although the results from our single-dose BD pilots are inconclusive on manipulating these levels in BD, Tie2 preserving strategies may be another interesting target in attenuating BD induced inflammation since we observed a profound reduction in Tie2 mRNA in renal tissue of BD rats and a trend in reduced Tie2 protein expression in BD mice. Before testing drugs that prevent Tie2 loss, the marked decrease in BD rat renal Tie2 expression needs to be confirmed in human DBD donors. Not only systemic donor pre-treatment but also blocking Ang2 induced processes via an Ang2 inhibitor or Ang1 derivate during the preservation period may be beneficial aiming to preserve/maintain organ viability/quality.

In conclusion, this thesis demonstrates that the Ang/Tie2-system is quite dynamic before, during and after renal transplantation, especially in the DBD donor. The endothelial activation in hemodialysis, kidney donor and transplant recipient may have been reflected by the altered angiopoietin levels. However, the altered angiopoietin activation pathway may participate in the further deterioration of endothelial function in dialysis and transplant patient thereby shifting this system from biomarker to causative factor in vascular damage. Overall our studies reveal that there is a need for further therapeutical intervention studies in the angiopoietin/Tie2 pathway in experimental and cell culture conditions. These may ultimately pave the way for human Ang/Tie2-therapy studies in dialysis and transplant recipients.

FUTURE PERSPECTIVES

The potential of measuring Ang2 as marker for organ quality at time of donation needs to be determined in a clinical study including living and deceased donors and assess its sensitivity and specificity versus other markers. Until then it remains unclear if the associations we describe would translate into one of clinical impact. Moreover, a quick, validated clinical applicable Ang2 test should be developed before clinical implementation would be possible. Furthermore, the predictive potential of Ang2 on transplant outcome should also be investigated in other organ transplants. Given the widely described dynamics of the Ang/Tie2-system and the possibility to manipulate its effect by several intervention strategies, angiopoietins may not only serve as predictive or quality markers, but also as intervention target. Specifically, elucidating the Ang/Tie2-mechanism and its role in BD is further study worth. Generating more understanding of the molecular mechanisms that affect the Ang/Tie2-system would support study design in humans to ultimately translate to the clinical setting. Especially in defining future therapeutic approaches to inhibit endothelial dysfunction via the Ang/Tie2-system in hemodialysis, the DBD donor and renal transplant recipients.

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Nederlandse samenvatting

Voor patiënten met ernstig chronisch nierfalen is nierfunctievervangende behandeling noodzakelijk. Niertransplantatie is hiervoor de meest effectieve behandeling. Vergeleken met dialyse resulteert transplantatie in minder mortaliteit en een betere kwaliteit van leven. In niertransplantatie zijn donornieren afkomstig van levende of postmortale (overleden) donoren. Postmortale donornieren kunnen afkomstig zijn van hersendode oftewel deceased brain dead (DBD) donoren of van een donor die overleden is aan een hartstilstand, een zogenaamde deceased circulatory dead (DCD) donor. Hoewel nieren afkomstig van levende donoren een betere functie en graft survival (transplantaat overleving) hebben dan nieren afkomstig van postmortale donoren, zijn ze maar beperkt beschikbaar. Ondanks dat de acceptatiecriteria voor postmortale donoren de afgelopen jaren verruimd zijn, is de wachtlijst voor het ontvangen van een donornier de laatste jaren nauwelijks afgenomen. Jaarlijks overlijden honderden patiënten met chronisch nierfalen terwijl ze op de wachtlijst staan omdat er niet op tijd een donornier beschikbaar is. Het is daarom van groot belang het donoraanbod, de orgaankwaliteit en de transplantatie-uitkomst te verbeteren.

Nieren afkomstig van levende donoren hebben een betere graft survival vergeleken met postmortale donornieren. Om het donoraanbod zo volledig mogelijk te kunnen gebruiken zijn er interventies nodig, bijvoorbeeld door hersendood geïnduceerde ontsteking te verminderen, om de transplantatie-uitkomst te verbeteren. De beschreven rol van het Angiopoietine/Tie2-systeem ter plaatse van het endotheel in sepsis en endotoxemie wekte onze interesse gezien de rol van endotheelactivatie in dialyse, de DBD donor en andere aspecten van niertransplantatie.

Angiopoietines zijn groeifactoren die een belangrijke rol spelen bij de vaatnieuwvorming, angiogenese. De receptoren waar ze aan binden, Tie1 en Tie2, bevinden zich op het endotheel, de binnenste bekleding van de bloedvaten. Het endotheel speelt een belangrijke rol in de uitwisseling van stoffen tussen bloed en weefsel. Daarnaast komt het als eerste in contact met circulerende pathogenen. Endotheelactivatie en disfunctie speelt een belangrijke rol bij bloedstolling, inflammatie en verschillende ziektebeelden zoals sepsis. Het is aangetoond dat het Ang/Tie2-systeem een belangrijke rol speelt bij het behouden van de geïnactiveerde ruststaat van het endotheel. Van de vier beschreven angiopoietines is de werking van angiopoietine-1 (Ang1) en angiopoietine-2 (Ang2) het beste gekarakteriseerd. Ang1 en Ang2 binden beide aan Tie2 op een competitieve manier en met vergelijkbare affiniteit. Ang1 wordt continue aangemaakt door pericyten en gladde spiercellen en binding aan Tie2 induceert phosphorylatie wat leidt tot anti-inflammatoire signalen. Daarnaast voorkomt Ang1 op die manier celdood van het endotheel. De competitie door Ang2 voorkomt Ang1 binding aan Tie2 waardoor de inflammatoire respons vergroot wordt en er plasma lekkage ontstaat.

In gezonde individuen is er minimale expressie van Tie2 door endotheelcellen. Weibel Palade bodies, opslagplaatsen in het endotheel, slaan Ang2 op en geven het snel af indien zich een inflammatoire stimulus voordoet. De functies van Ang2 zijn complexer en minder duidelijk dan die van Ang1 en lijken meer afhankelijk te zijn van de context. Eerder klinisch en experimenteel onderzoek heeft aangetoond dat Ang2-geinduceerde endotheelactivatie een belangrijke rol speelt bij inflammatie, atherosclerose en ernstige ziekte zoals sepsis.
Aangezien endotheelactivatie een belangrijke rol speelt binnen nierfunctievervangende therapie, hebben we in dit proefschrift het Ang/Tie2-systeem onderzocht in dialyse, donatie en transplantatie.
In **hoofdstuk 2** hebben we Ang1 en Ang2 levels onderzocht in 100 hemodialysepatiënten. In het eerste uur van dialyseren zagen we een forse toename van Ang2. Daarnaast was Ang2 geassocieerd met markers van inflammatie en hartschade. Prospectieve analyse liet zien dat Ang2 levels geassocieerd zijn met mortaliteit en cardiovasculaire events. Ang1 bleek niet geassocieerd te zijn met klinische parameters of uitkomst na dialyse. Dit sluit aan op eerder onderzoek naar Ang2 in dialyse dat aantoonde dat Ang2 levels correleren met de mate en ernst van coronaire hartziekten en perifeer arterieel vaatlijden. Endotheelactivatie en disfunctie is een kenmerk van patiënten met chronisch nierfalen en patiënten die hemodialyseren. Aangezien endotheeldisfunctie zelf een oorzaak is van Ang2 afgifte door Weibel Palade bodies, speculeren we dat de verhoogde predialyse Ang2 levels een reflectie zijn van endotheelactivatie. Dat wordt onderbouwd door de sterke associatie tussen Ang2 en pro-endotheline levels die we in deze patiënten vonden. Het ophelderen van de verantwoordelijke mechanismes zou als basis kunnen dienen voor toekomstig interventieonderzoek naar het Ang/Tie2-systeem in hemodialyse.
Hoewel er onderzoek is gedaan naar circulerende angiopoietine levels in niertransplantatieontvangers en levende en postmortale donoren, is er nog weinig bekend over de angiopoietine respons tijdens een gehele niertransplantatiecascade, van donor tot ontvanger. Aangezien het is aangetoond dat Ang2 het snelst reageert in pathologische omstandigheden en daarnaast in verschillende ziektebeelden van voorspellende waarde is, hebben we zowel circulerend als arterioveneus Ang2 vanuit de nier onderzocht in niertransplantatie na levende donatie in **hoofdstuk 3**. In zowel de donoren als ontvangers veranderde plasma Ang2 gedurende de operatie vergeleken met de preoperatieve levels. Reperfusie had geen effect op de renale Ang2 afgifte. Dit is in tegenstelling tot een andere studie waar bij levende en DBD transplantatie de renale Ang2 afgifte toenam tijdens reperfusie. Gezien het gebruik van dezelfde assay is een technische verklaring voor dit verschil onwaarschijnlijk. Mogelijk zijn de levende donor populaties niet vergelijkbaar qua mate van endotheelactivatie voorafgaand en tijdens nefrectomie. Om de verschillen tussen transplantatiecentra wat betreft de selectie van levende donoren en

endotheelactivatie gedurende nefrectomie verder te verduidelijken is multicenter Ang2 analyse nodig. De prospectieve associatie tussen circulerend Ang1 en Ang2 met graft failure en mortaliteit in niertransplantatieontvangers is onderzocht in hoofdstuk 4. Dit is in lijn met eerder onderzoek waarin Ang2 geassocieerd is met inflammatoire processen en hartschade markers. Ook is het consistent met eerder gepubliceerd onderzoek in een ander cohort van niertransplantatieontvangers waarin Ang2 positief correleerde met CRP. Plasma Ang2 bleek hoger te zijn in niertransplantatieontvangers na postmortale donatie vergeleken met ontvangers van een levende donor. In de niertransplantatieontvangers na postmortale donatie was Ang2 geassocieerd met graft failure en mortaliteit. Dit wordt mogelijk verklaard door een pre-inflammatoire staat van de ontvangers wat bij een risico op orgaanfalen verergerd, weerspiegeld door Ang2-gestuurde endotheelactivatie. Bij niertransplantatie is het grootste aantal donornieren afkomstig van DBD donoren. Hoofdstuk 5 zoomt in op de morfologische en histopathologische schade in niertransplantatiebiopten veroorzaakt door hersendood. Hoewel onze onderzoeksgroep en anderen de nadelige effecten van hersendood op orgaankwaliteit en graft survival hebben aangetoond is het nog steeds moeilijk om de orgaankwaliteit op moment van donatie vast te stellen. Verschillende pretranplantatie scorings- en predictiemodellen aan de hand van verschillende histopathologische parameters zijn onderzocht. De resultaten hiervan hebben niet tot een eenduidige benadering geleid. Naast de klassieke schademarkers waren wij met name geïnteresseerd in de lymfevatdensiteit (LVD). Hoewel het belang van LVD in de ontwikkeling en progressie van nierziekten en –transplantatie overtuigend is aangetoond, is het nog niet goed onderzocht in pretransplantatie biopten en hersendood. Zoals verwacht lieten pretransplantatie biopten van hersendode donoren een toename in LVD en profibrotische, vasculaire en inflammatoire schade zien in vergelijking met biopten verkregen van levende donoren. Binnen de DBD groep was LVD gecorreleerd met tekenen van vaatlijden zoals hyalinose en met markers van interstitele schade zoals interstitiele fibrose. Of de toename in LVD de oorzaak of een gevolg is van de vasculaire schade is onbekend. Het zou kunnen dat pathologische verdikking van de vaatwand de normale vasculaire functie inclusief het vloeistoftransport belemmert en daarmee resulteert in een toename van de LVD. Validatie van LVD in het vaststellen van de pretransplantatie nierkwaliteit en de evaluatie of een apart scoringssysteem voor DBD donoren nodig is zal moeten worden vastgesteld in prospectieve analyse. In hoofdstuk 6 werd de endogene rol van Ang2 single nucleotide polymorphisms (SNPs) in de postmortale donor en ontvangers van niertransplantatie onderzocht. De associaties tussen Ang2 SNPs en de ontwikkeling van acute longschade en een toename van het risico op acute respiratory distress syndrome (ARDS) zijn naar aanleiding van eerder onderzoek gepubliceerd. Deze resultaten samen met de mogelijkheid van veranderde Ang2 eiwit expressie en effect onder verschillende Ang2 SNPs, en de mogelijke rol

van Ang2 in postmortale donatie en niertransplantatieontvangers, resulteerden in deze genetische Ang2 studie in een niertransplantatie cohort. Ang2 tagging SNPS waren geassocieerd met death censored graft survival bij analyse in zowel donor als ontvanger. Er werd geen associatie met mortaliteit aangetoond. Om de rol van genetisch Ang2 te bevestigen zal de functionaliteit van deze SNPs aangetoond moeten worden aan de hand van replicatieonderzoek. Een overzicht van de hersendoodpathofysiologie en de nadelige effecten op potentiële donornieren is beschreven in **hoofdstuk 7**. We beschrijven de huidige kennis rondom deze onfysiologische status, het effect daarvan op de nierkwaliteit, maar ook potentiële herstelmechanismes en de relevantie daarvan voor de transplantatie-uitkomst. Intracraniële bloeding of traumatische hersenschade zijn meestal de oorzaak van cerebrale compressie wat leidt tot irreversibele cerebrale schade. Uiteindelijk hernieert de hersenstam waardoor de herstenstamreflexen verloren gaan, er een Cushing reflex ontstaat door de catecholamineafgifte, gevolgd door hypotensie en uiteindelijke volledige circulatoir arrest. Door de complete disfunctie van de hypothalamus-hypofyse-as krijgen de meeste patiënten diabetes insipidus als gevolg van het niet meer vrijkomen van ADH (anti-diuretisch hormoon). Kort na het ontstaan van hersendood circuleren er hoge concentraties proinflammatoire cytokines zoals interleukine-6. Hersendood resulteert in endotheelactivatie en toegenomen influx van polymorphonuclear neutrophils (PMNs) en macrofagen in nier, lever en darm. De inflammatoire respons op orgaanniveau vraagt om inverventiestrategiën om de graft kwaliteit en survival na DBD donatie te kunnen verbeteren. Het frequent voorkomen van endotoxemie in DBD donoren is aangetoond. Endotoxemie beïnvloedt het Ang/Tie2-systeem door een toename in de vasculaire permeabiliteit te veroorzaken.
Een verstoorde Ang1/Ang2 ratio ten gunste van Ang2 is geassocieerd met een toename in mortaliteit gedurende sepsis terwijl een ratio ten gunste van Ang1 de vasculaire integriteit behoud en de inflammatoire response dempt. De endotoxemie samen met de overeenkomsten in pathofysiologie tussen hersendood en sepsis maken het Ang/Tie2-systeem een interessant interventiedoel om de nadelige consequenties van hersendood te verminderen. Experimentele studies met recombinant en Ang1 derivaten zoals recombinant humaan Ang1 (rhAng1) en Vasculotide hebben tot veelbelovende resultaten in sepsis in muizen en het beschermen van de volwassen vaten tegen plasmalekkage geleid. Daarom hebben we manipulatie van het Ang/Tie2-systeem onderzocht in drie proof of principle experimenten via twee Ang1 verhogende strategieën en met behulp van een Ang2 remmend antilichaam. In **hoofdstuk 8** onderzochten we de effecten van rhAng1, Vasculotide en de Ang2 remmer AMG 386 in een experimenteel hersendood rat model om op die manier de hersendode donor te simuleren. Hersendode ratten werden behandeld met rhAng1 of een placebo en vergeleken met sham-geopereerde ratten. In een tweede experiment werden hersendode ratten behandeld met Vasculotide of

een placebo. Noch rhAng1, noch Vasculotide behandeling beschermde tegen hersendoodgeïnduceerde inflammatie in rattennieren. Dit zou verklaard kunnen worden door een doserings- of timingprobleem. Mogelijk is Ang1 verhogende therapie voordeliger tijdens hersendood zelf, zodat er onmiddellijke competitie met toegenomen Ang2 plaats vindt. De gebruikte doseringen zijn gebaseerd op eerdere sepsis experimenten in muizen. Hoewel we het verschil in metabolisme en lichaamsgewicht in overweging hebben genomen zouden de gebruikte doseringen te laag kunnen zijn geweest. Daarom kunnen we een potentieel anti-inflammatoir effect bij een hogere dosering niet uitsluiten. Real-time PCR liet een opmerkelijke afname in Tie2 mRNA expressie zien in de hersendode ratten. Dit zou een andere mogelijke verklaring kunnen zijn waarom het Ang/Tie2-systeem niet gunstig werd beïnvloed door exogene toediening van deze Ang1 derivaten. Onze analyses laten geen afdoende resultaten zien of het Ang/Tie2-systeem daadwerkelijk werd beïnvloed ten gunste van Ang1 bij deze doseringen, al maken de mRNA expressie resultaten dit hoogst onwaarschijnlijk.

Hoewel de meerderheid van de preklinische studies over pathofysiologische condities die vergelijkbaar zijn met hersendood voornamelijk Ang1 verhogende interventiestrategieën hebben gebruikt, is de recente literatuur meer gefocust op Ang2 als de meest dynamische component van het Ang/Tie2-systeem in endotheelactivatie. Er zijn veelbelovende resultaten gepubliceerd over anti-Ang2 therapie in verschillende preklinische modellen waaronder een studie over hart allografts uit ratten. Daarom hebben we in een derde experiment onderzocht of Ang2-remming hersendoodgeïnduceerde inflammatie zou kunnen verminderen in het hersendood rat model. Deze studie was opgezet als een pilot met een enkele dosering van een anti-Ang2 antilichaam welke geen invloed had op systemisch Ang2. Daarnaast vonden we geen afname in systemisch of renale inflammatie in de hersendode ratten behandeld met dit antilichaam vergeleken met hersendode ratten die behandeld werden met een placebo. Door middel van een doseringstitratie studie met exogeen Ang1 of een Ang2-remmer zouden we kunnen achterhalen welke doseringen gebruikt zouden moeten worden in toekomstige experimenten. Hierna zou een experiment waarin Ang/Tie2-interventie in een hersendood tijdsreeks onderzocht wordt, ons in staat stellen definitievere conclusies omtrent een mogelijke rol van het Ang/Tie2-systeem in hersendood te trekken. Ofschoon de resultaten van onze pilots over Ang/Tie2-interventie in hersendood geen definitieve conclusies geven over Ang/Tie2-manipulatie in hersendood, zou Tie2 behoud een ander interessant doel kunnen zijn om hersendoodgeïnduceerde inflammatie te verminderen aangezien we een aanzienlijke afname in Tie2 mRNA expressie vonden in hersendode ratten en een trend in verminderde Tie2 eiwitexpressie in BD muizen. Voordat medicijnen die Tie2-afname voorkomen verder onderzocht worden dient de afname in renale Tie2 expressie bevestigd te worden in humane DBD donoren. Niet alleen systemische

behandeling van de donor maar ook Ang/Tie2-manipulatie gedurende de preservatieperiode zou voordelig kunnen zijn om de orgaankwaliteit te behouden en de functie in de ontvanger te verbeteren.
Alle resultaten van de hierboven beschreven studies zijn samengevat en worden bediscussieerd in **hoofdstuk 9**. Dit proefschrift laat zien dat het Ang/Tie2-systeem aan veranderingen onderhevig is voor, gedurende en na niertransplantatie, vooral in de hersendode donor. De endotheelactivatie in hemodialyse, nierdonor en -ontvanger zou weerspiegeld kunnen zijn door de veranderde angiopoietinelevels. De veranderde activatie van angiopoietinepathways zou echter een rol kunnen spelen in de verdere achteruitgang van endotheelfunctie in dialyse en transplantatie. Daarbij verandert de rol van dit systeem mogelijk van biomarker naar oorzakelijke factor in vasculaire schade. Onze studies laten zien dat verder therapeutisch interventieonderzoek naar het Ang/Tie2-systeem in (celkweek)experimenten nodig zijn. Deze zouden uiteindelijk de basis kunnen vormen voor de opzet van klinisch Ang/Tie2-therapie onderzoek in dialyse en niertransplantatieontvangers.

Author affiliations

S Assa[1]
SJL Bakker[1]
E van den Berg[1]
J van den Born[1]
MH de Borst[1]
MLC Bulthuis[2]
J Damman[3]
MCRF van Dijk[2]
CFM Franssen[1]
H van Goor[2]
BG Hepkema[4]
JL Hillebrands[2]
HS Hofker[3]
RM Jongman[2]
C Krikke[3]
HGD Leuvenink[3]
M van Meurs[2,5]
GJ Navis[1]
J Niesing[3]
GJ Nieuwenhuijs-Moeke[3,5]
PJ Ottens[3]
RJ Ploeg[3,6]
WG van Rijt[3]
MA Seelen[1]
H Snieder[7]
MMRF Struys[5]
H Tent[1]
ZJ Veldhuis[3]
NJ de Vries[3]
S Yazdani[2]
R Westerhuis[1]
J Wiersema-Buist[3]

1 – Department of Nephrology, University Medical Center Groningen, University of Groningen, the Netherlands
2 – Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, the Netherlands
3 – Department of Surgery, Groningen Transplant Center, University Medical Center Groningen, University of Groningen, the Netherlands
4 – Department of Transplant Immunology, University Medical Center Groningen, University of Groningen, the Netherlands
5 – Department of Anesthesiology, University Medical Center Groningen, University of Groningen, the Netherlands
6 – Nuffield Department of Surgical Sciences, John Radcliff Hospital, University of Oxford, United Kingdom
7 – Department of Epidemiology, University Medical Center Groningen, University of Groningen, the Netherlands

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Op de een of andere manier kregen mijn oud-huisgenoten in Groningen het idee dat er in het UMCG slechts één groot lab is waar bovendien meer geouwehoerd wordt dan daadwerkelijk aan de wetenschap wordt gewerkt. Ik heb ze uitgelegd dat vrijwel elke afdeling een eigen onderzoekslaboratorium heeft maar dat er inderdaad slechts één lab is dat bedoelt kan worden met de door mij gebruikte benaming te weten 'HET' lab.
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Dankzij mijn oud-collega's van LifeLines viel er in tijden van tegenslag ook heel veel te lachen. Ik hoefde de buitendeur maar te openen of de deuntjes van een (boyband)hitje kwamen me over de gang al tegemoet. Jullie gevatte humor is heel behulpzaam geweest.

Al stond ik er ten tijde van de lotingsuitslag niet zo enthousiast tegenover, mijn coschappen in Nij Smellinghe, Drachten zijn een gedenkwaardige tijd geweest.

Ik wil daarvoor met name de afdelingen Urologie, Orthopedie en Chirurgie bedanken voor de leerzame tijd op OK en de leuke (beoordelings)gesprekken met bijkomende adviezen. Mijn semi-artsstage heb ik met veel plezier in Delft en Rotterdam kunnen doen waarvoor ik Dr. R.M. Bloem, de volledige afdeling Orthopedie van het Reinier de Graaf Gasthuis en daarnaast drs J.H.J.M. Bessems van het Sophia Kinderziekenhuis/Erasmus MC graag wil bedanken.

Na Groningen en Rotterdam had ik me geen leukere ANIOS-plek kunnen wensen dan bij de Chirurgie in het Flevoziekenhuis. Ik wil graag de volledige afdeling bedanken voor de afgelopen leerzame en leuke maanden. Vooral voor de mogelijkheid om voor aanvang eerst mijn proefschrift zoveel mogelijk af te ronden. Ik kijk uit naar de komende tijd.

Leuke Lex, held. Je kwam geheel onverwacht als heerlijk enthousiaste spring in 't veld nog meer gezelligheid brengen tijdens mijn tijd in Rotterdam. Ik had het niet willen missen en ik weet je te vinden! Lieve Madelien, lieve Madels, er zijn nooit veel woorden nodig voordat je begrijpt wat ik bedoel. Ik geniet van de manier waarop je in het leven staat. Laat Londen merken dat je er bent en doe wat je moet doen. Big time.

Team paranimfpower. Ik weet dat dit geen Nederlands woord is. Het dekt gewoon zo lekker de lading. Met de grootste glimlach en een enkel traantje denk ik terug aan alle momenten die we samen gedeeld hebben. Ik ben ontzettend blij en trots dat jullie als me als paranimfen bijstaan. De energie die ik kreeg van de fantastische momenten samen hebben als belangrijke basis voor dit proefschrift gediend. Lieve, fantastische Pau. En met fantastisch bedoel ik dat met evenveel lading als tijdens je eigen 21-diner speech. Heel veel prachtige herinneringen hebben we opgebouwd, zowel in als buiten het ziekenhuis. Onze connectie binnen het ziekenhuis hadden we voor de intensiteit van onze vriendschap niet nodig maar het extra begrip dat je daardoor had is van grote waarde geweest. Ik heb me gelukkig geprezen dat ik jou nog extra jaren dicht in m'n buurt had. Alleen al je unieke en hilarische oneliners vormden een grote energiebron. Dank voor al je steun, het luisteren en het feit dat je er altijd voor me bent.

Lieve Roos. Mooi mens met 'n groot hart. Timing is alles. Toen Pau en ik voor de zoveelste keer aan de tocht Amsterdam-Groningen moesten geloven wist jij op precies het goede moment het enige juiste bericht te sturen. Met je eindeloze energie en enthousiasme weet je altijd het onderste uit de kan te halen. We hebben meer dan eens een teleurstelling voor ogen gehad maar samen hanteerden we 'It ain't over 'till it's over' als motto in de 'strijd', of het nou om onderzoeks- of coschapperikelen ging of het oplossen van Oegandese/'This is Africa'-frustraties.

Gelukkig heeft Fleur ook in mijn belang gehandeld en woon je nu lekker dichtbij in Amsterdam. Tot in de laatste eindfase heb je me van een energieboost voorzien, en nog eens extra op onverwachtse momenten. Heel veel dank voor je trouwe onvoorwaardelijke steun en het zijn zoals je bent.
De combinatie van parallel coschappen lopen & promoveren/sociaal leven is niet altijd makkelijk geweest. Naast onderzoek in Groningen hadden we alle drie nog meer plannen voor ogen waardoor we meer uren in de trein doorbrachten dan misschien gezond is. Ook bij deze gemeenschappelijke deler zijn jullie van onschatbare waarde geweest. In de zin van vriendschap maar ook in het creëren van mogelijkheden en oplossingen. Tegen jullie allebei wil ik zeggen: ik kijk uit naar al onze plannen voor de toekomst.

LINKerds, mooie mensen. Ik ken geen groep die zo goed in staat is de ruimte te geven aan zoveel verschillende individuen, die in haar waarde te laten, en tegelijkertijd zo'n sterk en terugkerend groepsgevoel neer te zetten. Het is heerlijk om te zien hoe dit na meer dan tien jaar nog steeds lukt. We hebben momenten gedeeld waarin we tot het uiterste gehoopt en gehuild maar ook maximaal gelachen hebben. Ik geniet van jullie zoals jullie zijn. In elk gesprek is er ruimte voor meerdere verhaallijnen tegelijkertijd. Met of zonder volume, over het kammetje of op de top, op de momenten dat het kan of dat je moet. Houd dit vast en let daar op. Doe wat je kan en meer. Dank voor het steeds maar weer creëren van leuke herinneringen op momenten van ontsnapping en afleiding. Stuk voor stuk zijn jullie op een moment op een eigen manier van grote steun geweest. Waarschijnlijk vaker onbewust dan bewust. Live a little en blijf dat doen. Ali, Bob, Clara, Foz, Hef, Birk, Ireen, Jootje, Monk, Laut, Leo, Lies, Lais, Lo, Loes en Sop. Samen.

'Vrij kletsen'. Een term die ik in ons huis leerde en nog steeds graag gebruik. Lief huis, ook jullie hebben me een fantastische studententijd bezorgd waarbij het gelukkig niet is gebleven. Dansen, verkleedpakken nieten (ja dat werkt vrij goed), stuca's bouwen en ludieke plannen smeden, ik denk er met veel plezier aan terug. Lieve Hoop, ik heb veel aan je adviezen gehad en daarnaast was jij degene die de route Groningen-Amsterdam voor jaar '04 ophelderde. Ik begrijp het nu. Lieve Ien, ik heb genoten van je gezelligheid en je eigenschap om alles ook eens in een ander daglicht te plaatsten. Lieve Puck, je hebt een heerlijke nuchtere kijk op zaken. Leuk om ook nu steeds te horen dat en vooral hoe je alles regelt. Lieve Jel, al is m'n eigen energielevel even niet wat het wezen moet, jij straalt altijd voor twee. Moedige en dappere Nellie, je staat al jaren hoog op m'n 'opschep-lijst'. Je maakt ons trots, ook als je eventueel je doelen bijstelt, het maakt niet uit. Lieve, lieve Lo. Er is zoveel gebeurd sinds die eerste week in Groningen. Ik was zo blij met de raakvlakken die we toen ontdekten en gelukkig zijn die er, nu ik je weer in de buurt heb, nog. Ik bewonder hoe je in staat blijft werk en doorstuderen te combineren en dat je

van je passie en talent je werk hebt gemaakt. Nog even en we kunnen bij jou ook de afronding vieren. Maat, lieve Maat. Een typische krijs en een duo spreekbeurt houden over… tja, laat maar. Naar de plaatselijke bar-bodega-disco-dancing of juist niet, in beide hoedanigheden ben je een grote steun geweest. Je tomeloze drive is inspirerend, ik ben trots op de weg die je hebt afgelegd. Lieve Pien, dank voor je opgewektheid en het non-stop dansen. Een nummer missen is gewoon zonde. Ons hele huis, maar vooral jij begrijpt dat. Lieve Lot, je bokst zoveel voor elkaar en bent bereid daar ook hard voor te werken. Heel knap hoe jij je doelen voor ogen weet te houden en ook bereikt. Al is het nu op afstand, je houdt onze vriendschap hecht. Lieve Eef. Samen met Pau (en Roos) heb je gedurende mijn laatste jaren in Groningen het verschil gemaakt. Je heerlijke spot on gevoel voor humor en zorgzame, lieve karakter is een combinatie om trots op te zijn. Lieve Jol, al was het slechts een jaar, ik ben blij dat ik dat jaar dagelijks van je oneliners en energie heb kunnen genieten. Je doorzettingsvermogen is bewonderingswaardig. Daar ga je het ver mee schoppen. Ook wil ik al m'n oudere en jongere oud-huisgenoten bedanken. Dank voor jullie interesse, steun, het ophalen van herinneringen waar we nóg om kunnen lachen en jullie moeite om elkaar op te blijven zoeken.

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Over de auteur

Welmoet Hillegonda Westendorp werd geboren op 7 februari 1985 te Den Haag. Op vierjarige leeftijd verhuisde zij naar Driebergen-Rijsenburg. In 2004 behaalde ze haar Atheneumdiploma aan het Montessori Lyceum Herman Jordan te Zeist waarna ze een jaar Farmacie studeerde aan de Rijksuniversiteit Groningen. In 2005 werd ze alsnog ingeloot voor de studie Geneeskunde aldaar. Tijdens een cursus van de Junior Scientific Masterclass (JSM) op Schiermonnikoog werd haar interesse voor wetenschappelijk onderzoek naar orgaantransplantatie gewekt. Hierop volgend deed zij twee JSM proefprojecten op het Chirurgisch Onderzoekslaboratorium en een onderzoeksproject via de International Federation of Medical Students' Associations op Malta. Hierna begon zij aan haar Wetenschappelijke Stage op het Chirurgisch Onderzoekslaboratorium in combinatie met enkele cursussen. Vervolgens werd zij aangenomen voor een MD/PhD-traject onder supervisie van prof. dr. H.G.D. Leuvenink, prof. dr. H. van Goor en prof. dr. R.J. Ploeg. Gedurende dit traject combineerde ze haar coschappen met onderzoek wat uiteindelijk tot dit proefschrift heeft geleid.

Welmoet deed haar junior coschappen in het Universitair Medisch Centrum Groningen (UMCG) en haar senior coschappen in Nij Smellinghe, Drachten en het Medisch Centrum Leeuwarden. In 2013 werkte ze in het kader van haar coschap Sociale Geneeskunde twee maanden bij de NGO Teso Students Development Organization in Kumi, Oeganda. Haar semi-artsstage deed ze grotendeels op de afdeling Orthopedie van het Reinier de Graaf Gasthuis te Delft (opleider dr. R.M. Bloem). Gedurende haar studie was Welmoet actief binnen enkele commissies en werkte zij onder andere bij Pharma Bio Research en UMCG LifeLines. Momenteel is Welmoet werkzaam als arts-assistent op de afdeling Chirurgie van het Flevoziekenhuis te Almere (opleider dr. P.C.M. Verbeek).